UNIVERSITY OF ALBERTA

PERMISSIVE CYTOMEGALOVIRUS INFECTION IN CULTURED VILLOUS TROPHOBLASTS

BY

DENISE GAYLE HEMMINGS

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY.**

DEPARTMENT OF MEDICAL MICROBIOLOGY AND IMMUNOLOGY

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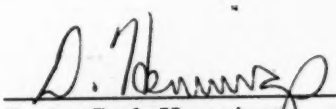

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ABSTRACT

Human cytomegalovirus (HCMV) is the leading cause of viral congenital diseases in the world resulting in high societal costs. Unlike rubella or toxoplasma infections, HCMV can be vertically transmitted even in mothers seropositive prior to pregnancy. Since 30 to 80% of urban women fall into this group, how this virus reaches the fetus and potential preventative therapies are relevant investigations. Placental infections are frequently associated with congenital infections suggesting hematogenous transmission, but exactly how HCMV crosses the placenta is not known. Specifically, the role of the syncytiotrophoblast (ST) as a barrier to infection or damage that could lead to vertical transmission is unknown. I hypothesize that HCMV is vertically transmitted by direct infection of villous ST followed by infection of underlying fetal stromal cells.

I initially investigated whether cultured primary trophoblasts could be permissively infected by HCMV. Compared to fibroblasts, I found that trophoblast infection was permissive but inefficient, required high viral inoculum, proceeded slowly and progeny virus remained predominantly cell-associated. I found reduced HCMV susceptibility in ST-like cultures with slow infection progression and little progeny virus production compared to cultures resembling cytotrophoblasts. Further investigation revealed that HCMV susceptibility was dependent on a relatively immature trophoblast maturation state and independent of the culture conditions.

Trophoblasts were cultured to tight-junctioned confluence on semi-permeable membranes to allow access to both apical and basolateral membranes. I observed multiple cell layers similar to the situation found *in vivo*, suggesting the relevance of this model to investigate disease transmission and maternal to fetal substance transfer. These

cultures were infected with HCMV and compartmentalization of progeny virus was monitored. Most virus remained predominantly cell-associated with little apical release and even less found in the basal compartment.

A model is developed of the proposed interactions at the level of the ST. Progeny virus is either retained by the ST or blocked by the trophoblast basement membrane. My original hypothesis, that infection alone results in HCMV vertical transmission, is unlikely to be true. However, the indirect ramifications of infection such as cell loss and maternal immune responses may lead to ST damage and subsequent fetal infection.

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ABBREVIATIONS

AEC	Aminoethylcarbazole
Caco-2	Colon epithelial cell-derived cell line
CT(s)	Cytotrophoblast(s)
ddH_20	Double-distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
Dpm	disintegrations per minute
EF-1α	elongation factor -1α
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EM	Electron microscopy
EVT	Extravillous trophoblasts
FBS	Fetal bovine serum
FcRn	Neonatal Fc receptor
GFP	Green fluorescent protein
GPCMV	Guinea pig cytomegalovirus
hCG	Human chorionic gonadotropin
HCMV	Human cytomegalovirus
HEL	Human embryonic lung fibroblasts
HIV-1	Human immunodeficiency virus type 1
HLA	Human leukocyte antigens

HPL	Human placental lactogen
HSV-1	Herpes simplex virus type 1
HTLV-1	Human T cell leukemia-lymphoma virus type 1
IE	Immediate early protein
IFN(s)	Interferon(s)
IMDM	Iscove's modified Dulbecco's medium
IVIG	Intravenous immune globulin
MDCK II	Madin-Darby canine kidney II epithelial cells
MEM	Minimal essential medium
MIEP	HCMV major immediate early promoter
MOI	Multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NF-κB	Nuclear factor kappa B
Ni-DAB	Nickel-enhanced diaminobenzidine
NK	Natural killer cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PF	Placental fibroblasts
PFU	Plaque-forming unit
PLAP	Placental alkaline phosphatase
PMNL	Polymorphonuclear leukocyte
RPE	Retinal pigment epithelial cell
RT	Room temperature

SD	Standard deviation
SEM	Scanning electron microscopy
ST	Syncytiotrophoblast
TBM	Trophoblast basement membrane
TdT	Terminal deoxynucleotidyl transferase
TEM	Transmission electron microscopy
TER	Transepithelial electrical resistance
TNF-α	Tumour necrosis factor alpha
TUNEL	TdT-mediated dUTP-biotin nick end labeling
Vim	Vimentin

CHAPTER 1.0 INTRODUCTION

1.1 PROLOGUE

The clinical manifestations of infections acquired *in utero* range from mild or no apparent illness to severe sequalae or death by resorption, abortion or stillbirth. Neonates with a silent infection at birth may experience late-onset disease such as the hearing loss often associated with rubella or cytomegalovirus (HCMV) infections or chorioretinitis associated with *Toxoplasma gondii* infections. Understanding the mechanism(s) by which pathogens are transmitted from mother to fetus could ultimately lead to successful prophylactic and therapeutic strategies and reduce high annual treatment costs. Any investigation of vertical transmission (transmission of a pathogen from mother to fetus) requires an understanding of placental and membrane development during pregnancy and their potential barrier functions so a brief overview is presented. Infectious agents could potentially reach the fetus through a number of routes prior to the rupture of fetal membranes and these routes are briefly described. For many pathogens the hematogenous route (across the villous placenta via mother's blood) is thought to be predominant, although the actual mechanism of transmission is largely unknown.

I chose to investigate the mechanism of vertical transmission by human cytomegalovirus (HCMV) because it is currently the leading cause of congenital viral infections in the world and results in a significant number of children with long-term sequalae including sensorineural deafness. The societal economic burden of caring for and supporting these children is considerable. The second half of this introduction will therefore concentrate on a description of HCMV, its involvement in congenital infections and what is currently known about the relationship between HCMV placentitis and vertical transmission in both humans and the guinea pig model.

1.2 PLACENTAL PHYSIOLOGY

A brief overview of placental physiology is presented to give the reader a background into the origin and features of the villous trophoblast. For a more thorough treatment of placental development, the reader is directed to an excellent resource by Benirschke and Kaufmann (Benirschke, Kaufmann, 2000d).

1.2.1 Placentation

Implantation of a rapidly dividing human blastocyst occurs within a week of conception and is potentiated by the trophoblasts that completely surround the embryoblast. Upon contact with the endometrial epithelium, the trophoblast cells fuse and form the continuous non-proliferating multinucleated layer called the syncytiotrophoblast (ST) that eventually completely surrounds the blastocyst. Trophoblasts not in contact with maternal cells remain mononuclear, proliferate and form a second layer of cells called the cytotrophoblasts (CTs; Boyd, Hamilton, 1966; Benirschke, Kaufmann, 2000b). The trophoblasts at the position of implantation eventually form the placenta and the rest of the trophoblasts surrounding the embryo transform into the smooth chorion and membranes.

Formation of the placenta begins with division of the trophoblasts at the implantation pole into three layers: the primary chorionic plate, the lacunar system and the trophoblastic shell (reviewed in Benirschke, Kaufmann, 2000b). The primary chorionic plate is composed of a layer of embryoblast-derived mesenchymal cells facing the blastocystic cavity that are covered by layers of CTs and ST facing the lacunae (Enders, King, 1988). The lacunar system is composed of vacuoles formed by pillars of ST called trabeculae. The vacuoles or lacunae eventually fill with maternal blood when disintegration of endometrial blood vessels by increasing trophoblast invasion occurs. The trabeculae are invaded by CTs from the primary chorionic plate and are always separated from the lacunae by a continuous layer of ST (Benirschke, Kaufmann, 2000b). The stems of the villous trees are formed when mesenchymal cells from the extraembryonic mesenchyme layer and fetal capillaries begin to invade these double-layered structures (Boyd, Hamilton, 1970).

The trophoblastic shell facing the endometrium is formed by fusion of the trabeculae and is initially composed of ST (Anonymous, 1941; Boyd, Hamilton, 1970). Deeper invasion of the endometrium is accomplished once the CTs have reached the trophoblastic shell with maternal spiral artery erosion leading to effective maternal blood circulation within the placenta (Boyd, Hamilton, 1970; Pijnenborg *et al.*, 1981a). In response to trophoblastic invasion, the endometrial stroma cells begin to proliferate and give rise to enlarged decidual cells (Welsh, Enders, 1985). Trabeculae that maintain

contact with the trophoblastic shell are not invaded with mesenchyme, remain predominantly trophoblastic and are called anchoring villi. Cytotrophoblastic cell columns within the anchoring villi are the sites of further longitudinal growth into the endometrium. The floating villi with fetal blood supply are extensions of stem villi originating in the chorionic plate and are the predominant sites of maternal fetal exchange. The lacunar system is now referred to as the intervillous space where the floating villi bring fetal circulation into close contact with maternal blood. The chorionic plate embedded with villous trees and the umbilical cord supplying fetal circulation and the basal plate resting on the placental bed are defined as the inner and outer borders of the placenta and enclose the intervillous space. The extensive system of villi are anchored in places through the anchoring villi in the basal plate. The chorionic and basal plates at the placental edge fuse to become the chorion laeve underlying the fetal membranes (reviewed in Benirschke, Kaufmann, 2000b).

Extravillous trophoblasts (EVT) are defined as any trophoblasts existing outside of the placental villi and include those in the basal plate, the chorionic plate, the chorion laeve and the marginal zone (Frank, Kaufmann, 2000). Within the basal plate, extravillous trophoblasts can be found in the cell columns, septa and cell islands. Two phenotypes of EVT exist: the proliferative phenotype are found closest to villous basal lamina and likely represent the EVT stem cells; the invasive phenotype including the endovascular trophoblasts that remodel the spiral arteries are found deeper in the decidua, are non-proliferative and represent a more differentiated EVT (Frank, Kaufmann, 2000). These two phenotypes can be distinguished by differential expression of growth factor receptors such as epidermal growth factor receptor (EGFR; c-*erbB*-1) and c-*erbB*-2 (Muhlhauser *et al.*, 1993; Duello *et al.*, 1994; Jokhi *et al.*, 1994), colony-stimulating factor-1 receptor (c-*fms*; Jokhi *et al.*, 1993) and endoglin (part of the transforming growth factor-β receptor complex; Caniggia *et al.*, 1997). An integrin switch from the basal lamina receptor α6β4 which is found predominantly in the proliferative phenotype to the interstitial extracellular matrix receptors such as α5β1, α1β1 and αv also define the two EVT phenotypes (Damsky *et al.*, 1993; Zhou *et al.*, 1993). A novel cell surface molecule (HIP) that attaches to heparan sulphate proteoglycans such as perlecan is found in cytotrophoblasts and only weakly in syncytiotrophoblasts. The interaction between HIP

and HSPGs was found to be essential for an EVT invasion phenotype (Rohde *et al.*, 1998). A molecule that promotes cell-to-cell adhesion, E-cadherin, is turned off in invasive EVT (MacCalman *et al.*, 1998) while expression of matrix metalloproteinases (MMPs) are upregulated in order to degrade the endometrial extracellular matrix during trophoblast invasion (Graham, Lala, 1991; Bischof, Martelli, 1992). Finally, the invasive phenotype can be distinguished from the proliferative phenotype by the expression of nonclassical non-polymorphic major histocompatibility complex (MHC) molecules including HLA-G, HLA-C and HLA-E (Hutter *et al.*, 1996). The function of these molecules is debated but one likely role is the inhibition of NK cell activity against trophoblasts deep in the basal plate (Brooks *et al.*, 1999; Le Bouteiller, Blaschitz, 1999). Trophoblast invasion may terminate by fusion of the EVT into multinucleated trophoblastic giant cells, which are found deep within the myometrium (Boyd, Hamilton, 1970; Pijnenborg *et al.*, 1980b; Winterhager *et al.*, 1999). These multinucleated cells can be distinguished from those of the villous syncytiotrophoblast by their expression of nonclassical HLA molecules and integrin expression (Chumbley *et al.*, 1993; Loke *et al.*, 1997).

A hemochorial placenta is thus formed where maternal blood is in direct contact with fetally derived trophoblasts. In the first two trimesters there are two complete layers of trophoblast (hemodichorial), which decreases for the most part to one layer by third trimester (hemomonochorial; Enders, 1965). To gain access to the fetus, pathogens originating in maternal blood must first cross the trophoblast layers and then other placental components including the trophoblastic basal lamina, the villous stroma containing connective tissue and the fetal endothelium with its basal lamina. Both the distance and the number of potential barriers between maternal and fetal blood reduces as pregnancy progresses: from 50 to 100 μm and the abovementioned barriers in first trimester to as low as 4 or 5 μm and four barriers (ST, trophoblast basal lamina, endothelial basal lamina and fetal endothelium) by term (Benirschke, Kaufmann, 2000b). A brief description of each placental component that could act in part as a barrier is given below.

1.2.2 Syncytiotrophoblast

The ST is a multinucleated layer that completely lines the intervillous space and all villi (reviewed in Castellucci, Kaufmann, 2000). It is continuous without cell borders and where gaps occur due to degeneration or damage, a fibrin-type fibrinoid is often found. The brush border or apical membrane in direct contact with maternal blood differs in expression and concentration of enzymes, proteins and receptors with the basal membrane that faces the trophoblast basal lamina. These differences likely relate to the ultimate function of the ST as the "decisive barrier that limits or supports transplacental transfer processes" (Benirschke, Kaufmann, 2000). The 12 m^2 surface area of the villous ST brush border is increased by a factor of 7.67 when the microvilli that almost completely cover this surface are taken into account (Teasdale, Jean-Jacques, 1986). In early pregnancy the ST is characterized by a homogenous distribution of nuclei and organelles (Boyd, Hamilton, 1970). After 15 weeks, areas differing in thickness, nuclei distribution, enzymatic activity and organelle composition can be detected within the continuous syncytium (Castellucci, Kaufmann, 2000). These areas of syncytiotrophoblastic specialization are briefly described to emphasize the variability and dynamic properties of this cell layer.

1.2.2.1 Vasculosyncytial membranes

In terminal villi enlarged fetal sinusoids can be found pushed up directly against the trophoblastic basement membrane (Fox, Agrafojo-Blanco, 1974). At this point, all nuclei and most organelles within the ST have been displaced leaving thin lamellae (0.5 to 1 μm) called epithelial plates or vasculosyncytial membranes. The basement membranes of the ST and that supporting the fetal endothelial cells come into close contact and may fuse (Castellucci, Kaufmann, 2000). In the term placenta this type of specialization constitutes 25 to 40% of the terminal villous surface area (Sen *et al.*, 1979). The physical structure and increased activity of enzymes involved in glucose transfer but not energy metabolism, suggests the role of vasculosyncytial membranes is efficient gas, glucose, amino acid and electrolyte transfer (Castellucci, Kaufmann, 2000).

1.2.2.2 Areas with predominantly rough endoplasmic reticulum

At term the most common area of ST has a thickness of 2 to 10 μm, is densely covered with microvillae and contains many organelles such as mitochondria with extensive rough endoplasmic reticulum and Golgi (Castellucci, Kaufmann, 2000). Detection of a large variety of enzymes, proteases and hormones by immunohistochemistry suggest that this area of ST is likely the major site of protein and lipid synthesis and complex transfer mechanisms (Castellucci, Kaufmann, 2000).

1.2.2.3 Areas with predominantly smooth endoplasmic reticulum

Small areas within the syncytium near tubular-like mitrochondria resemble active endocrine cells with a predominance of smooth endoplasmic reticulum (Gillim *et al.*, 1969; Crisp *et al.*, 1970). Detection of 3β and 17β-hydroxysteroid dehydrogenase specifically in these areas suggest that they may be specific sites of steroid hormone production (Castellucci, Kaufmann, 2000). Thin syncytial lamellae that are often extensions of the vasculosyncytial membranes but cover CTs also demonstrate specific immunohistochemical staining for 17β-hydroxysteroid dehydrogenase (Castellucci, Kaufmann, 2000).

1.2.2.4 Areas of concentrated nuclei

Benirschke and Kaufmann (Benirschke, Kaufmann, 2000a) suggest that areas of ST containing accumulated nuclei should be named according to function as described in this section. They have proposed that the terms **syncytial knots** or **Tenney-Parker changes** should be used to describe areas in third trimester ST with aggregated nuclei that are detected as a result of artifact from two-dimensional flat sectioning of irregular villous surfaces (Benirschke, Kaufmann, 2000a). In these areas, structural and histochemical features are similar to nearby normal ST and the nuclei show no apoptotic changes. Although not describing true apoptotic knotting, these changes could still be used as indicators of abnormal villous growth observed in various problematic pregnancies.

Areas with aggregations of small nuclei with highly condensed chromatin that demonstrate typical cytoplasmic degenerative changes should be referred to as **apoptotic**

knots (Cantle *et al.*, 1987; Benirschke, Kaufmann, 2000a). These include the slight bulges in syncytium often found near the vasculosyncytial membranes and areas that extend into the intervillous space. An estimated 150,000 apoptotic knots per day pinch off and enter the uterine venous blood but are only rarely found in peripheral blood suggesting removal by phagocytosis in the maternal lung (Ikle, 1961; Wagner, 1968). This apoptotic shedding can occur throughout pregnancy and is probably a result of normal trophoblast turnover (see below).

Benirschke and Kaufmann feel the term **syncytial sprout** should be reserved for large aggregates of nuclei that extend into the intervillous space in early pregnancy that represent further expansion of the villous tree (Castellucci *et al.*, 1990; Benirschke, Kaufmann, 2000a). These true sprouts are characterized as drum stick-shaped with large non-apoptotic nuclei. The majority of "syncytial sprouts" found in later pregnancy are due to tangential sectioning artifacts (Benirschke, Kaufmann, 2000a).

True **syncytial bridges** connecting neighboring villi by fusion of syncytiotrophoblastic surfaces after prolonged contact has been demonstrated (Burton, 1986; Cantle *et al.*, 1987; Burton, 1987). However, the majority of bridges detected in two-dimensional paraffin sections are due to tangential sectioning across villous branches (Burton, 1986; Cantle *et al.*, 1987). Whether true syncytial bridges are merely accidental or function in mechanical support is unknown (Castellucci, Kaufmann, 2000).

1.2.3 Cytotrophoblasts

Cytotrophoblasts or Langhans cells (named after their discoverer T.H. Langhans in 1882) are the stem cells feeding the ST layer by fusion. They form a complete second layer of trophoblast in early pregnancy that is reduced to approximately 20% by term, although the overall total number of CT continues to increase (Simpson *et al.*, 1992; Mayhew *et al.*, 1999). The reduction of this layer is important not only to increased maternofetal exchange through vasculosyncytial membranes but also to IgG transport since CTs do not express Fc-γ receptors (Bright *et al.*, 1994; Bright, Ockleford, 1995). Incorporation of ^{3}H-thymidine into CTs but not ST along with detailed ultrastructural studies has confirmed the stem cell function of CTs in renewal of the ST (Richart, 1961; Boyd, Hamilton, 1966).

Differentiation of CTs from the immature proliferating stem cell stage that is easily distinguishable from the ST to cells containing increased organelles and an electron density similar to ST occurs just prior to fusion. A recently fused cell remains distinguishable from the rest of the ST for a period of time in two aspects: the large oval mainly euchromatic nucleus and the high concentration of organelles (Castellucci, Kaufmann, 2000). Nuclear size is thought to reflect syncytial age with small nuclei in apoptotic knots being the oldest in degenerating areas (Martin, Spicer, 1973).

Production of human chorionic gonadotropin (hCG) and human placental lactogen (hPL) likely occurs once differentation of the CT stem cells takes place. In normal placental development this may occur just after or during fusion events. If fusion is delayed or non-existent as occurs in pathological conditions and in the extravillous trophoblast, secretion of these hormones may occur prior to fusion but at the same stage of differentiation (Castellucci, Kaufmann, 2000).

1.2.4 Trophoblast turnover and apoptosis

Recent studies by Huppertz et al suggest that trophoblast fusion is intimately related to apoptotic events that begin in CTs and are ultimately completed by the shedding of apoptotic knots from the syncytium (Huppertz *et al.*, 1999). A phosphatidylserine (PS) flip from inner to outer membrane leaflet is an early reversible apoptotic event (Martin *et al.*, 1995) that has been detected in differentiated CTs (Huppertz *et al.*, 1998) and is crucial to syncytial fusion (Lyden *et al.*, 1993; Adler *et al.*, 1995; Katsuragawa *et al.*, 1997). The concomitant presence of anti-apoptotic proteins such as Bcl-2 (LeBrun *et al.*, 1993; Sakuragi *et al.*, 1994; Kim *et al.*, 1995; Lea *et al.*, 1997; Marzioni *et al.*, 1998) and Mcl-1 (Huppertz *et al.*, 1999) may protect CTs against progression to irreversible apoptotic stages (Huppertz *et al.*, 1999). Fusion of these cells may renew degenerating syncytium by provision of mRNA for anti-apoptotic and other important proteins that it can no longer make itself since it appears to be transcriptionally inactive (Huppertz *et al.*, 1998). These specialized areas of the ST would then be protected from apoptotic progression and would be involved in active transport and hormone production. Focal areas of the syncytium without regions of CT fusion would progressively age and exhibit further signs of apoptosis until the extrusion of aged

TUNEL-positive nuclei and cytoplasm into apoptotic knots that are pinched off and shed into maternal uterine venous blood (Huppertz *et al.*, 1998; Huppertz *et al.*, 1999).

By roughly correlating ST weight gain over time with the CT proliferation and ST extrusion rates of nuclei, Huppertz et al determined that the majority of fusing CTs are not required for expansion of the ST, but are likely important for injection of fresh proteins and RNA not produced in the syncytium. An estimated 26 days between initial fusion events and extrusion of apoptotic nuclei suggests that the lifespan of in vitro cultures of ST is limited without fusion renewal (Huppertz *et al.*, 1998).

Evidence for trophoblastic turnover *in vivo* was shown by Palmer et al using first trimester organ culture (Palmer *et al.*, 1997). The ST is not a stagnant rigid barrier but a fluid cell layer that is constantly renewed by fusion of the underlying CT and repaired by placement of fibrin type fibrinoid in areas where mechanical damage or damage induced by apoptosis exposes the trophoblast basement membrane to maternal blood (Nelson, 1996; Burton *et al.*, 1996; Watson, Burton, 1998).

1.2.5 Trophoblastic basement membrane

Villous CTs secrete in a basal direction the components to form an electron dense trophoblast basal lamina or basement membrane (TBM) which consists of Type IV and V collagens, fibrinogen, laminin, entactin, heparan sulphate proteoglycan, fibronectin, occasional osmophilic droplets and multiple filaments 4 to 11 nm wide (Ashley, 1965; Bray *et al.*, 1975; Johnson, Faulk, 1978; Virtanen *et al.*, 1988; Onisto *et al.*, 1989; Nanaev *et al.*, 1993). Fibronectin may bind the TBM to the stromal connective tissue thus providing a support for the trophoblasts. Migration of the CTs to syncytiotrophoblastic sites requiring fusion may occur through the local activity of proteins interfering with this connection such as tenascin (Ekblom, Aufderheide, 1989; Castellucci *et al.*, 1991). This layer, an average of 20 to 50 nm thick, may also act as a filter by excluding large particles such as colloidal thorium dioxide but allowing smaller proteins such as ferritin to pass through (Ashley, 1965). Evidence for ongoing damage and repair mechanisms of the ST often exposing the TBM to maternal blood (Burton *et al.*, 1996) emphasizes its importance as a barrier to transplacental passage of pathogens and maternal cells (Burton, Watson, 1997).

1.2.6 Villous stroma

The villous stroma, separated from the trophoblasts by the TBM, contains three distinct types of mesenchymal cells or fibroblasts (reviewed in Castellucci, Kaufmann, 2000) that are defined primarily by shape, size and staining pattern for cytoskeletal antigens. All are vimentin positive. These cells are embedded in and are likely involved in the production of connective tissue fibers and various extracellular matrix components including collagens, laminin and heparan sulfate.

Also found within the villous stroma are fetally derived tissue macrophages first described in detail by Hofbauer in 1903 and referred to by that name (reviewed in Vince, Johnson, 1996). These are large vacuolated cells with granulated cytoplasm and are easily distinguished from other stromal cells. Hofbauer cells are phagocytic and like other tissue macrophages of the body carry Fc and C3b receptors. By second trimester they express Class II MHC implicating this cell type in antigen presentation to developing fetal lymphocytes.

1.2.7 Fetal endothelium

The final line of fetal defence are the mesenchymal-derived endothelial cells lining fetal blood vessels and additionally in later pregnancy the basal lamina supporting these cells (Demir *et al.*, 1989). Early in gestation small vessels form deep within the stroma, but by term the endothelial cells can be located directly adjacent to the ST where the basement membranes of each associate closely (Castellucci, Kaufmann, 2000).

1.3 ROUTES OF VERTICAL TRANSMISSION

Protection of the fetus from infection involves a three-way balance between maternal immune function, the placental barrier and fetal immune function, the first two being of paramount importance early in pregnancy. After 20 weeks gestation passive deliverance of maternal antibody in addition to the fetus' ability to mount an immune response provide additional protection (Nahmias, 1997). In order for a pathogen to successfully reach and infect the fetus, it must bypass, evade or cause an imbalance in these levels. Although I will limit my discussion to breaching or bypassing the placental barrier, these other equally important levels of protection should always be kept in mind.

Many pathogens are able to reach the fetus despite the presence of a placenta, demonstrating the lack of an absolute barrier function. Prior to rupture of the membranes, a number of potential routes of vertical transmission exist. The two most important and commonly known routes are the ascending route from genital tract to amniotic sac and the hematogenous route from maternal blood across the villous placenta. Other potential routes of infection include a) from infected endometrium or decidua to the trophoblastic cell columns, b) iatrogenically, c) from infected male gametes or d) from infected maternal peritoneum to the fallopian tubes.

1.3.1 Uterus, fallopian tubes or male gametes

Although vertical transmission of infections occurring through the fallopian tubes or through an infected spermatozoon at the time of fertilization is possible, documented evidence is lacking. Preexisting uterine infections could lead to infection of invading trophoblast cells during implantation but severe endometritis may prevent implantation (Fox, 1997). There is however some evidence for chronic endometrial gland infections in animals that do not affect fertility but may result in placental and fetal infection (Fox, 1997). Retrograde infection of the placenta through infection of the trophoblastic cell columns has been suspected in infections with hepatitis virus (Lucifora *et al.*, 1988; Lucifora *et al.*, 1990), HCMV (Fisher *et al.*, 2000) and toxoplasmosis (Benirschke, Kaufmann, 2000c). Evidence for HCMV DNA and mRNA transcripts in cervical smears and uterine glandular epithelial cells, interstitial tissue and muscle as well as persistent cervical secretion of infectious virus in non-pregnant women suggest that this route may be of some importance (Dehner, Askin, 1975; Wenckebach, Curry, 1976; Furukawa *et al.*, 1994). Studies looking at the incidence of HCMV infections in spontaneous abortions found that infection in the glandular epithelium of the decidua or the endometrial glands was associated with infection of the fetus (Dehner, Askin, 1975; van Lijnschoten *et al.*, 1994). As well, endometrial biopsies of non-pregnant women showed a 30% positivity rate for HCMV antigens by immunohistochemistry (van Lijnschoten *et al.*, 1994). However, other studies were unable to detect HCMV infection in abortions (Monif, Dische, 1972; Putland *et al.*, 1990; Cook *et al.*, 1993; Sifakis *et al.*, 1998) and in many

cases decidual infection could not be identified where chorionic villi were clearly infected (Boue *et al.*, 1966), confirming this is not the only route to fetal infection.

1.3.2 Invasive procedures

Each time an invasive technique is performed for diagnosis or therapy of the fetus *in utero*, the potential for transmission of infection exists. Although uncommon and certainly preventable, selected examples include HCMV infection (King-Lewis, Gardner, 1969; Evans, Lyon, 1991) and a gram-negative bacterial infection (Scott, Henderson, 1972) following intrauterine transfusion, bacterial (MacVicar, 1970; Haag *et al.*, 1974; Bastert *et al.*, 1975; Fray *et al.*, 1984; Hovav *et al.*, 1995; Ayadi *et al.*, 1998) and monilial (Elliott, 1984) infections following amniocentesis and Group B streptococcal sepsis following umbilical cord puncture (McColgin *et al.*, 1989).

1.3.3 Ascending from genital tract

An ascending infection occurs when organisms normally inhabiting or abnormally invading the lower genital tract of pregnant women enter the amniotic sac resulting in amniotic sac infection syndrome. Inflammation of the membranes (chorioamnionitis) is the predominant manifestation of an ascending infection that generally begins with a maternal leukocytic infiltration of extraplacental membranes and is usually followed by fetal leukocyte involvement after 20 weeks of pregnancy (Benirschke, Kaufmann, 2000c). The immune cells may in some cases be almost entirely fetal in origin (Sampson *et al.*, 1997) with clear evidence for an active fetal immunological defence against pathogens entering the amniotic sac from the genital tract (Dammann, Leviton, 2000). The placental villous tissue is either not involved or demonstrates significantly less infiltration than the membranes. While various bacterial, mycotic and mycoplasmic organisms have been isolated from inflamed placental membranes and amniotic fluid (McGregor, French, 2000; reviewed in Fox, 1997), only one virus, herpes simplex (Altshuler, 1974; Hyde, Giacoia, 1993; Benirschke, Kaufmann, 2000d), has been implicated in ascending infections.

In this route of transmission, the organism must first bypass the physical barrier of the intact membranes and then escape the antibacterial activity of the amniotic fluid

before successfully infecting the fetus. Inhalation or ingestion of infected amniotic fluid or direct infection of fetal skin or eyes are the usual entry points in fetal infections (Benirschke, Kaufmann, 2000c). The barrier presented by the intact membranes is likely mechanical in nature (Chany *et al.*, 1966) since there is no associated antibacterial activity (Talmi *et al.*, 1991) and bacterial penetration is only weakly prevented (Gyr *et al.*, 1994). Many studies demonstrate the ability of organisms to cross the intact membranes resulting in amniotic sac infection (Armer, Duff, 1991), although most are associated with premature rupture of the membranes (reviewed in Fox, 1997; Benirschke, Kaufmann, 2000c). Amniotic fluid particularly after 20 weeks gestation contains numerous substances that inhibit bacterial growth (Schlievert *et al.*, 1977; Gleicher *et al.*, 1979; Scane *et al.*, 1988). The risk of transmission to the fetus through an ascending infection is dependent on the type of organism and the time of infection onset but is independent of the extent of inflammation (Benirschke, Kaufmann, 2000c). Although these two barriers in combination must provide some protection since the incidence of fetal infection after chorioamnionitis is infrequent (Fox, 1997), it remains one of the common routes of fetal infection, particularly by bacteria, and may be a frequent cause of death (Lessing *et al.*, 1989).

1.3.4 Hematogenous transmission

Various pathogens may also be transmitted from maternal blood through the villous placenta to the fetus. Villitis, inflammatory lesions within the villous, is a common manifestation of hematogenously derived fetal infections with involvement of the membranes markedly less if present at all (Fox, 1997). Lesions may be focal involving only scattered villi, which is most commonly found, or diffuse involving many villi throughout the placenta. A number of bacteria present in maternal blood during infection can infect the placenta resulting in villitis that in some cases is associated with transmission to the fetus. These include *Treponema pallidum* (syphilis; Ohyama *et al.*, 1990; Grossman, 1977; Walter *et al.*, 1982; Qureshi *et al.*, 1993; Genest *et al.*, 1996), *Listeria monocytogenes* (Altshuler, Russell, 1975; Lessing *et al.*, 1989; Gersell, 1993), *Chlamydia psittaci* (Gunson *et al.*, 1983; Thoma *et al.*, 1997; Hyde, Benirschke, 1997; Jorgensen, 1997), *Campylobacter fetus* (Gribble *et al.*, 1981), *Brucella abortus* (Sarram

et al., 1974; Porreco, Haverkamp, 1974), *Mycobacterium tuberculosis* and *Mycobacterium leprae* (reviewed in Benirschke, Kaufmann, 2000c). The hematogenous route is how the majority of viral infections are thought to be transmitted to the fetus, i.e. through placental villi in contact with viremic maternal blood. Viruses that are likely transmitted to the fetus by this route include rubella (Tondury, Smith, 1966; Thompson, Tobin, 1970; Kohler, Bridson, 1971; Catalano *et al.*, 1971; Ornoy *et al.*, 1973; Garcia *et al.*, 1985; Cradock-Watson *et al.*, 1989), varicella (Garcia, 1963; Paryani, Arvin, 1986; Qureshi, Jacques, 1996; Benirschke *et al.*, 1999), enteroviruses (Hughes *et al.*, 1972; Garcia, 1982; Amstey *et al.*, 1988), mumps (Garcia *et al.*, 1980), parvovirus B-19 (Anand *et al.*, 1987; Samra *et al.*, 1989; Torok, 1994), hepatitis (Lucifora *et al.*, 1988; Lucifora *et al.*, 1990; Feucht *et al.*, 1996), human immunodeficiency virus (Backe *et al.*, 1994; Burton *et al.*, 1996; Benirschke, Kaufmann, 2000c) and HCMV (see Section 1.5). Fungal infections rarely cause placental infections and are unlikely to cross via a hematogenous route to the fetus. Parasites and protozoa such as *Toxoplasma gondii* (Elliott, 1970; Desmonts, Couvreur, 1974; Garcia *et al.*, 1983; Popek, 1992; Stray-Pedersen, 1993), *Trypanosoma cruzi* (Bittencourt, 1976; Gilson *et al.*, 1995) and *Plasmodia* (malaria; Walter *et al.*, 1982; Redd *et al.*, 1996; reviewed in Benirschke, Kaufmann, 2000c) may infect the placenta and be transmitted to the fetus via this route.

The protective barrier function of the placenta to blood-borne pathogens is evident in situations where placental infections without accompanying fetal infections are observed during maternal tuberculosis, syphilis, malaria, coccidioidomycosis, HCMV and rubella infections (Mims, 1968; Hayes, Gibas, 1971; Spark, 1981). However, this barrier function may only be partial since a large variety of known pathogens can cross the placenta albeit at varying rates of transmission and in some cases are only delayed by the presence of the placenta. Likewise, infection of the fetus can be observed even in the absence of demonstrable placental infections suggesting that mechanisms other than direct placental infection may result in passage of the pathogen (Remington, Klein, 1995; Benirschke, Kaufmann, 2000c). Even observations of a positive infection of villous stromal cells may not prove vertical transmission by the hematogenous route since the placenta could be affected after the virus is first disseminated from the fetus (Quan, Strauss, 1962).

The first cell in direct contact with maternal blood in the intervillous space is the ST of the floating villi. Possible mechanisms by which pathogens could cross this potential barrier include:

1. Infection of the ST with subsequent passage of infectious progeny to underlying cells that lead to fetal infection. Infection of the ST has been associated with fetal infection by *Chlamydia psittaci* (Hyde, Benirschke, 1997), rubeola (measles; Moroi *et al.*, 1991), rubella (Ornoy *et al.*, 1973), hepatitis B (Lucifora *et al.*, 1990), *Toxoplasma gondii* (Elliott, 1970; Benirschke, Kaufmann, 2000c), *Trypanosoma cruzi* (Bittencourt, 1976) and HCMV (see Section 1.5).
2. Damage to the ST layer by non-specific sheer mechanical forces or a damaging maternal immune response to infection could result in transplacental passage of the pathogen or infected maternal cells with subsequent fetal infection. Damage potentially leading to infection by HIV-1 has been investigated (Burton *et al.*, 1996a; Burton *et al.*, 1996b) and enhanced binding of activated maternal immune cells to syncytiotrophoblast and TNFα-mediated loss of syncytium has been demonstrated (Xiao *et al.*, 1997; Garcia-Lloret *et al.*, 1999).
3. Uptake of an antibody-bound pathogen through Fc receptors normally found on ST (Simister, Story, 1998). One pathway by which IgG molecules may pass through ST is via nonspecific fluid-phase endocytosis where they are rescued from degradation by binding to FcRn in endosomes at low pH (6.0) (Simister *et al.*, 1996; Kristoffersen, Matre, 1996) and then transferred through to the basal surface. Whether an antigen-antibody complex could bind to FcRn and be shuttled through in a manner similar to unbound IgG is unknown. As well, the low pH requirement for FcRn to bind IgG may inhibit some pathogens i.e. HCMV (Kempf *et al.*, 1991).
4. Migration of infected maternal cells through the ST. Transfusion of maternal cells to fetal circulation has been demonstrated (Walknowska *et al.*, 1969; el-Alfi, Hathout, 1969; Moszkowski *et al.*, 1971; Redline, Patterson, 1993) and

suggested as a mechanism in HIV-1 vertical transmission (Peuchmaur *et al.*, 1991).

5. Phagocytosis and transcytosis of organisms by ST. HIV-1 was shown to move by transcytosis through intact tight-junctioned epithelial cells and infect cells in a bottom culture chamber (Bomsel, 1997; Lagaye *et al.*, 2001).
6. Passage through transtrophoblastic channels. Evidence exists for the presence of pores or channels extending through the ST that are 15 to 25 nm in diameter that allow passage of small water-soluble molecules (Stulc *et al.*, 1969; Thornburg, Faber, 1977; Hedley, Bradbury, 1980; Kaufmann *et al.*, 1987). Conditions in which fetal venous pressure is elevated appear to cause dilation of the distensible channels so that all molecules regardless of size may pass through (Kertschanska *et al.*, 1997). Thus passage of infectious pathogens may occur by this route under certain conditions.

One should also bear in mind that actual passage of the pathogen to the fetus may not pose the greatest risk to fetal success. Widespread damage to the ST or the uteroplacental vessels could in itself result in reduced blood flow, endocrine function, transfer of nutrients and oxygen and removal of fetal waste products. For example, bacterial infection by *Coccidioidomycosis* causes infarcts and necrosis in the placenta that result in fetal complications, but the fetus is not infected per se (McCaffree *et al.*, 1978; Spark, 1981). Infection by HCMV has recently been shown to inhibit invasiveness of extravillous first trimester CTs (Fisher *et al.*, 2000) which could result in more serious pregnancy complications such as preeclampsia (Khong *et al.*, 1986), intrauterine growth retardation and preterm labor (Garcia, 1982; Bernstein, Divon, 1997). In view of the extensive reserve capacity of the placenta, however, unless this type of damage is severe, the overall functional ability of the placenta is unlikely to be seriously affected (Castellucci, Kaufmann, 2000).

1.4 CYTOMEGALOVIRUS

1.4.1 Description

Enlarged or "cytomegalic" cells with nuclear inclusions resembling owl's eyes were detected in association with stillborn infants as early as 1881 by Ribbert (Ribbert,

1904). The characteristic inclusions resembled those caused by varicella and herpes simplex infections thus suggesting a viral agent (Goodpasture, Talbot, 1921; Lipschutz, 1921). Isolation of the virus by three separate groups occurred in the early 1950s and the term cytomegalovirus was coined by Weller in 1960 (reviewed in Mocarski, 1996).

HCMV, a member of the subfamily *betaherpesvirinae* within the family of *Herpesviridae*, is a large ubiquitous species-specific double-stranded DNA virus. The virions are 150 to 200 nm in size and the linear DNA is encased by an icosahedral capsid. Between the capsid and the outer viral glycoprotein-studded envelope lies the tegument or matrix (reviewed in Mocarski, 1996). During a permissive infection with HCMV an equal amount of infectious virions and non-infectious "dense bodies" are produced (Craighead *et al.*, 1972; Sarov, Abady, 1975). Dense bodies are composed of a viral envelope surrounding pp65, a tegument protein, with no nucleocapsid or viral DNA (Irmiere, Gibson, 1983). Non-infectious enveloped particles containing a capsid but no viral DNA are also produced but less frequently than dense bodies (Irmiere, Gibson, 1983).

1.4.2 Growth Cycle

A fully permissive HCMV infection progresses to production of complete infectious progeny virus with host cell cytopathology. In an abortive infection viral proteins produced early in infection may or may not be expressed but no progeny virus is produced (Rice *et al.*, 1984; Weinshenker *et al.*, 1988; Cinatl *et al.*, 1994; Sinzger *et al.*, 1996; Gerna *et al.*, 2000). A latent infection occurs when the virus enters a state of quiescence and is essentially hidden within the cell. The mechanistic details of this latter type of infection are still largely unknown (Fish *et al.*, 1998)

HCMV appears to be transmitted primarily in a cell-to-cell manner (Navarro *et al.*, 1993). High levels of free and cell-associated HCMV in blood are generally found only in severely infected immunocompromised individuals (Gerna *et al.*, 1991). Usually, the levels of virus in blood as detected by viremia, pp65-antigenemia and leukoDNAemia tests are consistently low and in most cases of short duration even during primary infections in normal healthy individuals (Revello *et al.*, 1998).

Permissive infection with HCMV proceeds in a manner similar to HSV-1 (reviewed in Mocarski, 1996). HCMV initially binds with low affinity via glycoprotein B to heparan sulfate proteoglycans (Compton *et al.*, 1993) followed by higher affinity binding to secondary receptors which may include 92.5 kDa and 34 kDa cell surface proteins, CD13 or annexin II (Adlish *et al.*, 1990; Nowlin *et al.*, 1991; Soderberg *et al.*, 1993; Wright *et al.*, 1994; Compton, 1995; Fortunato, Spector, 1999). Receptors for HCMV are common to many cell types and HCMV can attach and enter nearly all cell types including those not permissive for viral replication (Fortunato *et al.*, 2000). Penetration is unlikely to occur by endocytosis and probably involves fusion of the viral envelope with the host cell membrane mediated by viral glycoproteins such as gH and gL (Compton *et al.*, 1992; Compton, 1995; Milne *et al.*, 1998). There is mounting evidence that mere attachment of infectious or non-infectious HCMV particles including dense bodies to cell surface receptors initiates a series of signal transduction cascades resulting in stimulation of arachidonic acid metabolism, increased reactive oxygen intermediates, sequence-specific DNA binding of NF-κB, the induction of Fos, Jun and Myc transcription factors and transient Ca^{2+} influx (Speir *et al.*, 1996; Speir *et al.*, 1998; Fortunato *et al.*, 2000).

Following penetration, viral nucleocapsids are transported directly to the nucleus likely via actin-mediated transport and the 230-kb viral DNAs are placed at nuclear domain 10 (ND10) sites. A number of cellular proteins that are interferon (IFN) inducible localize to these sites. Only the HCMV viral genomes found at the ND10 sites generate Immediate Early (IE) transcripts emphasizing the role they play in lytic infections (Ishov *et al.*, 1997; Maul *et al.*, 2000). Interestingly, HCMV infection or in fact glycoprotein B alone can induce the cell to produce antiviral IFN-α inducible cellular mRNAs (Boyle *et al.*, 1999). Once virus infection has progressed, however, the cell becomes unresponsive to IFN-α induced antiviral effects and a decrease in IFN-γ signal transduction also occurs (Miller *et al.*, 1999; Miller *et al.*, 2000).

HCMV replication, like other herpesviruses, is dependent on temporally regulated expression of viral genes: α or immediate early (IE) gene products regulate expression of β or delayed early genes and these early proteins are essential for expression of γ or late genes (reviewed in Mocarski, 1996). Unlike other viruses in this family, replication of

HCMV is slow, requiring up to 72 hours to produce progeny virus. Any restriction on permissiveness generally occurs after penetration and often involves a block in viral gene expression either before or just after IE-antigen expression producing an abortive infection (see references in Mocarski, 1996). Host cell DNA replication is not an absolute requirement for permissive HCMV infections (DeMarchi, Kaplan, 1977), however, confluent contact-inhibited cultures show reduced infection levels and decreased progeny virus production (DeMarchi, Kaplan, 1977).

Human CMV replicates *in vivo* in a variety of human cells including epithelial, endothelial, smooth muscle, neuronal, macrophage and glial cells (reviewed in Sinzger, Gerhard, 1996). *In vitro* it preferentially replicates in human fibroblasts (Smith, 1986; Poland *et al.*, 1990), although low levels of replication occur in other cell types (Michelson-Fiske *et al.*, 1975; Knowles, 1976; Tumilowicz *et al.*, 1985; Smith, 1986; Lathey *et al.*, 1990; Poland *et al.*, 1990), particularly if they have been treated with a differentiating agent (Tanaka *et al.*, 1985; Weinshenker *et al.*, 1988; Lathey, Spector, 1991; Ibanez *et al.*, 1991; Poland *et al.*, 1994).

Accumulation of nucleocapsids in the nucleus results in nuclear inclusions or the "owl's eye" cytopathic effect. Viral egress likely follows an exocytic pathway through the Golgi apparatus since it is sensitive to brefeldin A (Eggers *et al.*, 1992) and cells with poorly developed Golgi tend to release less virus (Mocarski, 1996). Release of 50% of progeny virus from infected fibroblasts into the supernatant late in infection demonstrates the efficiency of this process. Progression to cell lysis is evident for many cell types (Poland *et al.*, 1990; Kahl *et al.*, 2000) but not necessarily all (Michelson-Fiske *et al.*, 1975; Fish *et al.*, 1995; Fish *et al.*, 1998) and in some cases occurs but is greatly delayed (Tumilowicz *et al.*, 1985).

1.4.3 Transmission and epidemiology

In developed countries 40 to 60% of adults from upper to middle class areas are seropositive for HCMV with that number rising to 80% in lower socioeconomic areas. Almost all adults in developing countries are HCMV positive (Demmler, 1991). HCMV has been detected in all body secretions and can be horizontally transmitted by close or intimate contact (reviewed in Britt, Alford, 1996). Vertical transmission to the fetus also

occurs resulting in a fetal infection rate of 0.2% to 2.2% in the United States and is currently the most common congenitally acquired virus in the world (Stagno *et al.*, 1986; Yow *et al.*, 1988; Demmler, 1994). Congenitally infected infants and those who acquire HCMV early in life, often through breastfeeding (Reynolds *et al.*, 1973; Stagno *et al.*, 1980), can secrete copious amounts of virus for long periods of time usually with no detectible symptoms (Stagno *et al.*, 1975; Stagno *et al.*, 1980). Thus day care centers and other areas with concentrated populations of children present high risk situations for HCMV transmission (Pass, Hutto, 1986; Pass *et al.*, 1986; Pass *et al.*, 1990).

Evidence for HCMV transmission via a sexual route includes viral shedding in both male and female genital tracts (Lang, Kummer, 1975; Stagno *et al.*, 1975; Rasmussen *et al.*, 1995) and the correlation of HCMV seropositivity to age of first intercourse, number of sexual partners, presence of other sexually transmitted diseases and use of non-barrier contraception (Chandler *et al.*, 1985; Handsfield *et al.*, 1985; Fowler, Pass, 1991). One study investigated the probable transmission of HCMV from semen during coitus to a pregnant woman prior to delivery resulting in endocervicitis (Benirschke *et al.*, 1974).

Transmission of HCMV also occurs through blood products and organ transplantation. Removal of leukocytes from blood reduces the 2.4% risk of transmission per unit of blood, suggesting this cell type as the viral carrier. Immunocompromised patients including those with AIDS and transplantation recipients consistently shed HCMV at multiple sites in almost 100% of cases.

1.4.4 Latency and reactivation

HCMV, like all herpesviruses, enters into latency after the initial primary infection (reviewed in Sweet, 1999). During latency viral DNA but no infectious virus can be detected in many different tissues. Although studies are not yet conclusive, $CD34^+$ hemopoietic stem cells, $CD33^+$ dendritic and myeloid progenitor cells, peripheral blood monocytes and endothelial cells may be the major sites of latency in the human (Taylor-Wiedeman *et al.*, 1991; Minton *et al.*, 1994; Fish *et al.*, 1998; Hahn *et al.*, 1998). In addition polymorphonuclear leukocytes (PMNL) may be a source of infectious virus even though replication is limited to the IE stage (Gerna *et al.*, 2000).

How latency is maintained and what stimulates reactivation are poorly understood. A permissive infection may not be necessary for induction of latency suggesting that non-permissively infected cells may also enter latency (Bevan *et al.*, 1996). There is some evidence that IE may (Schrier *et al.*, 1985) or may not (Bitsch *et al.*, 1993) be expressed in some latently infected cells. One model in HSV latency suggests that IE-antigen expression occurs but at levels too low to activate the early genes (Kramer, Coen, 1995). Some as yet unknown event then upregulates the production of IE so that some early gene proteins are produced and the infection proceeds (Kramer, Coen, 1995). In latently infected granulocyte-macrophage progenitors novel unspliced transcripts from the IE region have been detected that are similar to the latency associated transcripts (LATs) found in latent herpes simplex virus infections (Kondo, Mocarski, 1995). There is at present no known function for these transcripts. Stimulation of latently infected undifferentiated cells with agents to induce differentiation results in IE expression and progression of infection (Taylor-Wiedeman *et al.*, 1991; Taylor-Wiedeman *et al.*, 1994). IE gene expression is repressed in some non-permissively infected cells by cellular DNA-binding factors such as MBF1 (Shelbourn *et al.*, 1989) and YY1 (Liu *et al.*, 1994) that bind to the IE enhancer region. Various cytokines, NF-κB and virion-associated proteins can overcome this repression and enhance IE production (reviewed in Sweet, 1999). Alternatively, activation of a latent infection in granulocyte-macrophage progenitors has been demonstrated in vitro by cocultivation with allogeneic PBMCs suggesting a mechanism for HCMV reactivation during transplantation (Soderberg-Naucler *et al.*, 1997).

1.4.5 Immune response

The importance of an active immune response to HCMV is clearly demonstrated by the severity of disease exhibited in immunosuppressed patients such as those with AIDS, transplant recipients and infants congenitally infected during a primary maternal infection. Natural killer (NK) cells present a first line of defence, the importance of which was demonstrated in a patient with an NK cell deficiency who developed a severe HCMV infection (Biron *et al.*, 1989). Investigations in mice with a mouse-specific CMV

(MCMV) confirmed the importance of an initial NK cell response to control this viral infection (reviewed in Tay *et al.*, 1998).

However, clearance of HCMV infections is dependent on a cellular immune response. The major target for $CD8^+$ cytotoxic (CTL) and $CD4^+$ proliferative responses is the matrix phosphoprotein, pp65 (UL83), carried in the virion itself (reviewed in Rasmussen, 1990). Adoptive transfer of specific CTLs may protect against posttransplant disease (Riddell *et al.*, 1992; Walter *et al.*, 1995).

A substantial humoral response to HCMV has been clearly documented and it appears to have a role in reducing the severity of disease rather than preventing or clearing the disease, particularly with reactivated virus (reviewed in Britt, Alford, 1996). Maternal immunity prior to pregnancy has been reported to protect the fetus from severe symptomatic congenital infection (Yeager *et al.*, 1981; Tanaka *et al.*, 1991; Fowler *et al.*, 1992), although recent evidence suggests this may not be the case (Boppana *et al.*, 1999). Infection in transplant patients from reactivated virus is less severe than that of a primary infection (Rubin, 1990). Pooled human gammaglobulin provides some protection against virulent disease in transplant patients (Zaia, 1993) emphasizing the importance of this arm of the immune response.

1.5 VERTICAL TRANSMISSION OF CYTOMEGALOVIRUS

1.5.1 Incidence

0.2 to 2.2% of all babies born alive in the United States (Stagno, 1995) and 0.42% in one Canadian report (Larke *et al.*, 1980) secrete HCMV at birth which is indicative of a congenitally acquired viral infection. Of these babies about 10% are immediately symptomatic often presenting with petechiae, thrombocytopenia, intrauterine growth retardation, hepatosplenomegaly, jaundice, pneumonia and various central nervous system problems including intracranial calcifications, microcephaly, sensorineural deafness and chorioretinitis (Demmler, 1991). Of the 90% congenitally infected babies that are asymptomatic at birth, 10-15% will develop late-onset sequalae usually involving loss of hearing (Pass *et al.*, 1980). In 1992 in the U.S., the annual cost of caring for children with symptomatic HCMV-related conditions was $1.86 billion (Yow, Demmler, 1992).

HCMV vertical transmission occurs in approximately 40% of mothers with a primary infection and unlike rubella or toxoplasmosis is also transmitted in 0.2 to 1.8% of mothers who are seropositive prior to pregnancy (Schopfer *et al.*, 1978; Stagno *et al.*, 1982). Transmission in this latter group was thought to be due to reactivated maternal virus (Nankervis *et al.*, 1984) but may in fact be due to reinfection with a HCMV isolate distinct from the resident latent virus (Boppana *et al.*, 2001). In 1979 Dr. Henry Balfour described CMV as "the troll of transplantation":

> "Trolls in Scandinavian folklore are demon-like figures who dwell in subterranean places or caves. I like to compare CMV with a troll because for most of us this devil is hidden well away. We usually acquire CMV as a mild or asymptomatic respiratory infection, although it may manifest itself as heterophil-negative infectious mononucleosis. The virus then goes underground, so to speak, hiding in salivary gland or renal tubular epithelial cells. If immunologic defenses are intact, CMV may never be seen again. But if the host's immunologic capabilities are compromised, as they are by the exogenous immunosuppression needed to prevent allograft rejection, CMV may emerge from latency, replicate, and cause tissue destruction" (Balfour, Jr., 1979).

During pregnancy, some alteration, be it immune or otherwise, signals the HCMV "troll" to reappear. Since the majority of women are seropositive prior to conception, it appears likely that this group contributes as much if not more to the overall incidence of congenital HCMV infections than women undergoing a primary infection (Yow *et al.*, 1988; Alford *et al.*, 1990). It was previously reported that the majority of severely affected babies are born to the 0.7% to 4% of susceptible mothers who seroconvert during pregnancy (Stagno *et al.*, 1982; Stagno *et al.*, 1986; Fowler *et al.*, 1992). This suggested that a preexisting maternal immune response to HCMV offered the fetus protection from serious disease (Stagno *et al.*, 1982; Stagno *et al.*, 1986) even though it did not completely prevent transmission (Stagno *et al.*, 1982). However, recent evidence shows no difference in the severity of infection in congenitally infected babies from mothers with primary infections or mothers with immunity prior to pregnancy (Boppana *et al.*, 1999). Boppana et al recently showed that severity of congenital infection in this latter group was correlated to reinfection with a distinct HCMV isolate rather than reactivation of the preexisting latent virus (Boppana *et al.*, 2001). Additionally, higher antibody levels of longer duration during a primary or reactivated infection in pregnancy

did not appear to protect against vertical transmission and in fact were associated with increased transmission and disease manifestation (Alford *et al.*, 1988; Boppana *et al.*, 1993; Boppana, Britt, 1995). However, although higher amounts of antibodies were present, mothers who transmitted to their fetuses appeared to have reduced neutralizing antibody titres and antibodies with reduced avidity (Boppana, Britt, 1995) suggesting that the affinity maturation of the antibody response may be important to prevention of vertical transmission. The gestational age of seroconversion does not appear to affect transmission rates (Griffiths, Baboonian, 1984; Stagno *et al.*, 1986; Yow *et al.*, 1988; Demmler, 1991), but transmission occurring as a result of a primary or reactivated infection during the first trimester may result in more severe fetal consequences (Ahlfors *et al.*, 1983; Griffiths, Baboonian, 1984; Stagno *et al.*, 1986; Demmler, 1991; Boppana *et al.*, 1993). Studies done in guinea pigs show that although transmission is increased if maternal infection occurs late in pregnancy, the fetus is more severely affected if infection occurs early in pregnancy (Kumar, Prokay, 1983).

Other risk factors for vertical transmission of CMV include viral load (Stagno *et al.*, 1982; Stagno *et al.*, 1986; Griffith *et al.*, 1990), poor maternal lymphocyte proliferation response (Gehrz *et al.*, 1981; Stern *et al.*, 1986; Fernando *et al.*, 1993) that may be related to host genetic factors (Fitzgerald, Shellam, 1991; Curtsinger *et al.*, 1994) and socioeconomic status (Stagno *et al.*, 1982; Stagno *et al.*, 1986). A combination rather than any one single factor appears to be important to vertical transmission (Fernando *et al.*, 1993). Early studies looking for risk factors reported increased maternal viral excretion as pregnancy advanced (Numazaki *et al.*, 1970; Montgomery *et al.*, 1972; Reynolds *et al.*, 1973). However, when viral excretion levels were compared to a non-pregnant population, it was discovered that viral excretion was in fact suppressed early in pregnancy followed by an increase to pre-pregnant levels late in pregnancy (Stagno *et al.*, 1975). Viral secretions from the cervix and/or urine do not appear to correlate to the incidence of congenital infection (Reynolds *et al.*, 1973; Stern, Tucker, 1973), although recent studies suggest a correlation may indeed exist (Nankervis *et al.*, 1984; Fernando *et al.*, 1993).

1.5.2 Placental and villous trophoblast involvement in vertical transmission of HCMV

It is evident that the placenta and not just the immune state of the mother is important with respect to transmission, not only from the less than 100% transmission rate, but also from many studies involving transmission in monozygotic versus dizygotic twins, with the latter often occurring in only one twin (reviewed in Ahlfors *et al.*, 1988). The role of the placenta and specifically the villous ST that is in direct contact with maternal blood in vertical transmission are unknown. There are three main routes by which HCMV could pass through the placenta to the fetus. Fisher et al recently described predominant CT infection with no accompanying ST infection in sections of anchoring and floating first trimester chorionic villi (Fisher *et al.*, 2000). One possible route of fetal infection could thus be by cell-to-cell infection of invasive CTs by infected uterine cells leading to infection of the anchoring chorionic villi (Fisher *et al.*, 2000). The two other probable routes are by damage or infection of the outer ST layer of the floating chorionic villi lying within the intervillous space during maternal viremia (Becroft, 1981; Stagno, 1995; Castellucci, Kaufmann, 2000). The following sections summarize the findings and describe the advantages and disadvantages of various methods employed in investigating of the role of the placenta and villous trophoblasts in vertical transmission of HCMV. Table 1.1 presents a comprehensive summary of the literature describing HCMV infection of trophoblasts.

1.5.2.1 Examination of the placenta

The most relevant way to study the importance of the villous placenta in vertical transmission of HCMV would be *in vivo*. Although manipulation during human pregnancy is unethical, the human placenta can be examined after delivery in the event of a pregnancy complicated by HCMV. Preferential examination of placentas from severely affected infants or stillbirths (Garcia *et al.*, 1989), however, limits interpretations of the role of ST since the effects of a placental infection that may have progressed for a long period of time are difficult to analyze retrospectively (Monif, Dische, 1972; Schwartz *et al.*, 1992; Muhlemann *et al.*, 1992; Sinzger *et al.*, 1993). Additionally, only a finite number of sections can be examined and small focal infections of ST could be missed

(Alford *et al.*, 1964). Nevertheless, the limited snapshot of placental and trophoblast involvement at the time of examination has resulted in important observations and detailed descriptions of HCMV-associated placentitis in all three trimesters of pregnancy.

Although there are examples of histologically unaffected placentas in the face of identifiable congenital HCMV infection (Wyatt *et al.*, 1950; Feldman, 1969; Hayes, Gibas, 1971; Muhlemann *et al.*, 1992), typical HCMV-complicated placentas show variable levels of villitis, vasculitis and focal necrosis (reviewed in Blanc, 1961; Garcia *et al.*, 1989). This variability may be related to stage of pregnancy at the time of infection, duration of infection prior to examination, maternal viral load or fetal-derived immune response (Benirschke *et al.*, 1974). Third trimester placentas, and the trophoblast in particular, rarely display the inclusion bodies characteristic of permissive HCMV infections (Quan, Strauss, 1962; Altshuler, McAdams, 1971; Monif, Dische, 1972; Mostoufi-Zadeh *et al.*, 1984; Garcia *et al.*, 1989; Muhlemann *et al.*, 1992; Sinzger *et al.*, 1993; Nakamura *et al.*, 1994). A limitation of histological analysis is that in an advanced phase of infection affected cells may break down and be unidentifiable (Quan, Strauss, 1962). Immunohistochemical analysis of sections from third trimester placentas of symptomatic infants or stillbirths revealed infrequent IE (Muhlemann *et al.*, 1992; Sinzger *et al.*, 1993; Schneeberger *et al.*, 1994) but not early nuclear (Muhlemann *et al.*, 1992; Schneeberger *et al.*, 1994) or late (pp150; Sinzger *et al.*, 1993) antigens in trophoblasts, suggesting abortive infections (Sinzger *et al.*, 1993). The majority of IE, E and late expressing cells appeared to be vimentin-positive fibroblasts with some positive endothelial and macrophage cells (Sinzger *et al.*, 1993). In light of the ability of this virus to enter a latent phase, detection by the above methods may not reveal infection. Use of *in situ* hybridization revealed HCMV DNA primarily in stromal cells and occasionally in the trophoblast of term placentas with chronic villitis (Sachdev *et al.*, 1990; Ozono *et al.*, 1997).

Placentas from first or second trimester abortions contain nuclear inclusions frequently in stromal cells (Schwartz *et al.*, 1992; Muhlemann *et al.*, 1992) and occasionally in trophoblasts (Garcia *et al.*, 1989). Expression of IE (Fisher *et al.*, 2000; Gabrielli *et al.*, 2001) and pp65 antigens (van Lijnschoten *et al.*, 1994; Gabrielli *et al.*,

2001) in the trophoblast indicate a permissive infection in these cells during the first half of gestation is possible.

1.5.2.2 Animal models

The use of animal models widens the window of observation and allows sequential studies and manipulation of variables such as viral load, infection at various times of gestation and immune response on rates of transmission. The intact placenta can be examined at any time point chosen and its pathology related to vertical transmission (Griffith *et al.*, 1985). Murine CMV has not been demonstrated to cross the placenta and as such is not a relevant model for vertical transmission of HCMV (Johnson, 1969). The rhesus monkey CMV model, although related closely to the human, has not been characterized fully (London *et al.*, 1986). The guinea pig provides an excellent model because placentation and stages of pregnancy are similar to that of the human (Davidoff, 1973; Leiser, Kaufmann, 1994) and guinea pig CMV (GPCMV) is transmitted during pregnancy resulting in congenitally infected offspring with characteristics similar to those found in HCMV infected infants (reviewed in Bia *et al.*, 1983). GPCMV has been isolated from the placenta during congenital infections (Griffith *et al.*, 1985; Goff *et al.*, 1987; Griffith *et al.*, 1990) and using immunohistochemistry and *in situ* hybridization, placental infection in guinea pigs was localized to the ST lying in the transitional zone between capillarized and non-capillarized areas (Griffith *et al.*, 1985; Griffith *et al.*, 1990).

The long gestation period, small litter sizes, expense and paucity of readily available resources and reagents (Griffith, Aquino-de Jesus, 1991) are a few of the drawbacks to investigations involving guinea pigs. As always there is the relevance of extrapolating results obtained in animal models to that of the human situation. CMV is a species-specific virus and although GPCMV is similar to HCMV, differences exist that may not allow direct application to humans (Griffith *et al.*, 1990; Mocarski, 1996).

1.5.2.3 Placental perfusion

Dual perfusion of isolated lobules of the human placenta has been used to investigate HCMV and other infectious diseases (Amstey *et al.*, 1988; Muhlemann *et al.*,

1995). The advantage of this system is the ability to assess the interaction of virus with a viable human organ that maintains its normal morphology (Ringler, Strauss, 1990). However, even when the culture system has been modified to allow preservation of tissues for up to 48 hours (Polliotti *et al.*, 1996), the length of time may not be sufficient to investigate permissive infection by a slow growing virus such as HCMV which requires up to 72 hours to replicate (Mocarski, 1996). In a study by Muhlemann et al dual perfusion was conducted for 9.5 hours with no detection of placental infection (Muhlemann *et al.*, 1995). Limited tissue viability and the expense of operating the perfusion apparatus (Ringler, Strauss, 1990) make this method untenable for studies involving placental HCMV infections. Perfusion studies have been, however, very effective in demonstrating intact placental barrier function to passive entry by HCMV, Coxsackie B-3 and ECHO-11 viruses (Amstey *et al.*, 1988; Muhlemann *et al.*, 1995).

1.5.2.4 Organ and explant cultures

Small pieces of first trimester chorionic villi cultured in alternating gaseous and liquid phases have been studied with respect to infection by HCMV. Positive infection of CT and ST was identified by immunohistochemistry and histological features and although no progeny virus was detected by cocultivation (Amirhessami-Aghili *et al.*, 1987; Amirhessami-Aghili *et al.*, 1989), increasing viral DNA levels were identified by dot blot analysis (Amirhessami-Aghili *et al.*, 1989). Although these cultures have been reportedly maintained for to 10 days with no apparent cell damage (Amirhessami-Aghili *et al.*, 1987; Amirhessami-Aghili *et al.*, 1989), a major limitation of this type of study is cell viability and most studies are of short duration only (Ringler, Strauss, 1990; Sooranna *et al.*, 1999). However, recent studies using explants from first trimester and term placentas demonstrated degeneration followed by functional regeneration of syncytiotrophoblast even after 7 and 11 days in culture respectively (Palmer *et al.*, 1997; Siman *et al.*, 2001).

If first trimester anchoring villi are dissected and placed in culture on filters coated with extracellular matrix, CT from the cell columns will invade the matrix and form explant cultures (Ringler, Strauss, 1990; Fisher *et al.*, 2000). Expression of IE antigen has been recently demonstrated primarily in the CT and rarely in the ST of

floating and anchoring villi in explant cultures from 7 of 12 placentas tested (Fisher *et al.*, 2000). Disadvantages of this method include degeneration of the syncytial layer (Palmer *et al.*, 1997) and possible contamination by other placental cells, which is particularly relevant with respect to investigations involving HCMV (Ringler, Strauss, 1990).

1.5.2.5 Choriocarcinoma cell lines

Studies involving parts of or the entire intact placenta are important for investigating cellular responses to viral infections in context, but do not allow dissection of the role of specific cells within the placenta (Ringler, Strauss, 1990). Trophoblast-like cell lines derived from placental malignant tumours such as choriocarcinomas or by viral transformation are often used to investigate trophoblast characteristics *in vitro*. The commonly used and most characterized choriocarcinoma cell lines are BeWo (Pattillo, Gey, 1968), JEG-3 (Kohler, Bridson, 1971) and Jar (Pattillo *et al.*, 1971). The advantages of using these cell lines rather than isolated primary cells include their ability to proliferate, ease of acquisition and handling and the relative homogeneity of cells in culture. All of these lines produce hCG and some steroids at generally lower levels than isolated primary trophoblasts (Ringler, Strauss, 1990). In addition to the usual caution required when extrapolating results acquired from transformed cells to normal cells, a disadvantage of using these cell lines is that their characteristics often reflect those of cultured purified first trimester trophoblasts rather than term trophoblasts and may be a better model for extravillous than villous trophoblasts (reviewed in Ringler, Strauss, 1990). It has been reported that the JEG-3 cell line is non-permissive for HCMV (Rosenthal *et al.*, 1981).

1.5.2.6 Isolated purified cytotrophoblasts

A number of procedures have evolved over the years to isolate trophoblasts from placental tissue originating with the work of Thiede (Thiede, 1960) who used trypsinization to disaggregate placental cells. The primary concerns addressed by each of these procedures have been purity, adherence properties and viability (reviewed in Bloxam *et al.*, 1997a). Additionally, development of differentiated ST-like cultures from the immature CT has been essential for studies of placental barrier function (reviewed in

Bloxam *et al.*, 1997b). The advantages of using purified and viable primary trophoblasts in culture include ease of manipulation and the ability to assign specific functions or characteristics to this specific placental cell. However, it must also be remembered that *in vivo* these cells exist in the context of many other cell types that likely influence their responses and that isolation procedures may result in altered characteristics (Ringler, Strauss, 1990). Method development of co-culture with other isolated placental cells will begin to decipher *in situ* influences on trophoblast behaviour.

There are only a handful of studies investigating the susceptibility of cultured trophoblasts to HCMV infection. An initial study demonstrated that epithelioid cells isolated by collagenase digestion of placentas were not permissive for HCMV but contaminating fibroblastic cells were (Rosenthal *et al.*, 1981). Highly purified term trophoblasts were found to express IE and E antigens after HCMV challenge but did not release virus into culture supernatants unless co-infected with either HIV-1 (Toth *et al.*, 1995b) or HTLV-1 (Toth *et al.*, 1995a). These results are compatible with the *in vivo* findings of infrequent non-permissive trophoblast infections at term. However, it remains difficult to explain the 40% transmission rate resulting from primary maternal infections or the more frequent indications of permissive infections in first trimester trophoblasts on the basis of such co-infections.

TABLE 1.1[a] Summary of studies investigating trophoblast infection with HCMV

Gestational Age	Assay system	Detection Method	Detection of HCMV infection		Reference
			Villous Trophoblasts	Villous Stroma	
25 wks stillborn	PS	IB	ND	++	(Blanc, 1961)
Term stillborn	PS	IB	ND	ND	(Quan, Strauss, 1962)
19 wk abortus	PS	IB	ND	++	(Altshuler, McAdams, 1971)
Term	PS	IB	ND	ND	(Hayes, Gibas, 1971)
		Viral culture	++		
Term	PS	IB	ND	ND	(Monif, Dische, 1972)
2nd trimester	PS	IB	ND	++	
14-16 wks abortus	PS	IB	ND	++	(Benirschke *et al.*, 1974)
32 wks		IB	ND	ND	
35 wks		IB	ND	ND	
Term		IB	ND	+/-	
Term		IB	ND	ND	
8 cases, term	PS	IB	+/-	4/8 ++	(Mostoufi-Zadeh *et al.*, 1984)
		Viral culture	4/8 ++		
Group 1: 25 wks ave, abortuses	PS	IB	Group 1: +	Group 1: ++	(Garcia *et al.*, 1989)
Group 2: 36 wks ave, stillborn		IB	Group 2: ND	Group 2: +	
Group 3: 40 wks ave, liveborn		IB	Group 3: ND	Group 3: +/-	
Additional placentas	PS	IF	26/76 placentas positive in villous tissue		
4 cases, 14-27 wks	PS	IB	ND	3/4 ++	(Schwartz *et al.*, 1992)
		ISH	ND	3/4 ++	
6 cases, 19-37 wks	PS	IB	ND	5/6 ++	(Muhlemann *et al.*, 1992)
		IHC	1/6 ++ in ST	5/6 ++	

Gestational Age	Assay system	Detection Method	Detection of HCMV infection		Reference
			Villous Trophoblasts	Villous Stroma	
6 cases, 20-39 wks	PS	IB IHC	ND 2/6 + (IE only)	6/6 ++ 6/6 ++ (IE, E and L)	(Sinzger *et al.*, 1993)
1st trimester abortions	PS	IHC	6/89 ++ (pp65)	ND	(van Lijnschoten *et al.*, 1994)
4 cases 30-38 wks	PS	IHC	ND	1/4 ++	(Nakamura *et al.*, 1994)
Quadruplets – only 3 examined at 36 wks	PS	IHC	++ (IE only)	++ (IE only)	(Schneeberger *et al.*, 1994)
1st trimester	Explants	IF Progeny virus Dot blot hybridization	++ (antisera), ND Increasing viral DNA over time	ND	(Amirhessami-Aghili *et al.*, 1987; Amirhessami-Aghili *et al.*, 1989)
1st trimester	PS Explants	IF IF	++ ++	++ ND	(Fisher *et al.*, 2000)
1st trimester	Explants	IHC, progeny	++ (IE, E, L)	++ (IE, E, L)	(Gabrielli *et al.*, 2001)
Term	Perfusion	IHC	ND	ND	(Muhlemann *et al.*, 1995)
1st trimester and term	Cultured	Viral DNA	ND	++	(Rosenthal *et al.*, 1981)
Term ST	Cultured	IF Progeny virus	++ (IE, E) Preinfection with HIV-1 required for detection of progeny virus	n/a	(Toth *et al.*, 1995b)
Term ST	Cultured	IF Progeny virus	++ (IE, E) Dual infection with HTLV-1 required for detection of progeny virus	n/a	(Toth *et al.*, 1995a)
Term ST	Cultured	IHC, progeny	++ (IE, E, L)	n/a	(Halwachs-Baumann *et al.*, 1998)

TABLE 1.1 Summary of studies investigating trophoblast infection with HCMV

a This table only includes studies which specifically looked for infection in trophoblasts.

b Abbreviations: PS = placental sections, IB = inclusion bodies, ND = not detected, ++ = readily found, + = detected with some searching, +/- = found by searching diligently, IF = immunofluorescence, ISH = in situ hybridization, IHC = immunohistochemistry, n/a = not applicable, IE = immediate early, E = early, L = late.

CHAPTER 2.0 RATIONALE AND HYPOTHESIS

2.1 HYPOTHESIS

Maternal HCMV infections result in congenital infections in 0.2–2.2% of all live births and is one of the most common causes of mental retardation and non-hereditary sensorineural deafness in children (Strauss, 1985; Stagno, 1995). Even though the percentage rate is relatively low, the long-term nature and considerable expense of this congenital disease suggests that mechanisms of prevention and protection must be addressed (Yow, Demmler, 1992). What makes this virus a particular problem in pregnancy is that it can be transmitted to the fetus in the face of preexisting immunity (Schopfer *et al.*, 1978; Stagno *et al.*, 1982). Although primary infections during pregnancy lead to a higher transmission rate than reactivated infections (40% compared to 1%), more pregnant women experience a reactivated infection than a primary infection so the contribution to the congenital infection rate is approximately the same for both groups if not higher (Reynolds *et al.*, 1973; Stagno *et al.*, 1977).

Since only 40% of pregnant women with primary HCMV infections give birth to infected infants (Stagno *et al.*, 1986), an effective fetal barrier, either physical or immunological, must exist. Although the pathogenesis of HCMV transmission to the fetus during pregnancy is unknown, congenital HCMV infections are commonly associated with chronic villitis (Sachdev *et al.*, 1990; Nakamura *et al.*, 1994) and infection of the placenta (Quan, Strauss, 1962; Altshuler, McAdams, 1971; Monif, Dische, 1972; Benirschke *et al.*, 1974; Mostoufi-Zadeh *et al.*, 1984; Garcia *et al.*, 1989; Sachdev *et al.*, 1990; Schwartz *et al.*, 1992; Muhlemann *et al.*, 1992; Sinzger *et al.*, 1993; Nakamura *et al.*, 1994). Passage through the placenta (Becroft, 1981; Benirschke, Kaufmann, 2000d), which may also act as a viral reservoir (Griffith *et al.*, 1985), could potentially occur in two directions: up through the cell columns in the anchoring villi or across the villous ST from maternal blood in the intervillous space.

Infection originating in the uterine wall could lead to infection of extravillous CTs involved in either interstitial or endovascular invasion. Infection of these cells could progress in a retrograde manner through the cell columns of anchoring villi to the chorionic stroma and eventually infect the fetus (Fisher *et al.*, 2000). This route may be

more of a possibility during a reactivated uterine infection than during a primary infection, although contact of the endovascular trophoblasts with maternal blood does occur.

Within the intervillous space lie the chorionic villi, which are covered with a continuous ST that acts as a barrier between maternal blood and fetal tissues and is likely involved in protection from or dissemination of virus across the placenta (Benirschke, Kaufmann, 2000d). The two most probable routes of transmission through the villous placenta via maternal blood are one, by damage of the ST resulting in gaps through which infected cells or virus could pass or two, by infection of the ST that spreads in a cell-to-cell manner and leads to fetal infection. Evidence of permissive HCMV infection of the ST in the guinea pig model (Griffith *et al.*, 1985; Griffith *et al.*, 1990) and the detection of infected trophoblasts in placentas from congenitally infected babies (Garcia *et al.*, 1989; Muhlemann *et al.*, 1992; Sinzger *et al.*, 1993), suggest that transmission by the latter route may occur. However, nothing is known of the origin, nature or consequences of such infections. Understanding how this usually innocuous virus crosses the placenta and developing methods to prevent or ameliorate damage to the fetus would be of great societal benefit by decreasing its impact on public health resources (Yow, Demmler, 1992).

My general research focus is to investigate the role of the villous trophoblasts in vertical transmission of HCMV and to develop a suitable culture model to test therapeutic approaches to limit transmission. **My hypothesis is that vertical transmission of HCMV occurs by direct infection of villous ST followed by infection of underlying stromal cells leading to fetal infection. Specifically, cultured primary villous trophoblasts can be permissively infected with HCMV, which leads to release of progeny virus in a basal direction.**

2.2 RATIONALE

2.2.1 Human cytomegalovirus

Of the many pathogens that are transmitted from mother to fetus, I specifically choose to study HCMV for the following reasons:

1. HCMV is endemic, infecting 40-80% of civilized urban populations and thus is a relevant problem in all areas of the world (Demmler, 1991). Many of the other pathogens may be regional such as Trypanosoma cruzi (Chagas' disease) largely found in South America (Bittencourt, 1976) or target one particular group of people such as *Campylobacter fetus*, a bacteria that causes abortion in cattle and sheep that can infect the human placenta as well (Gribble *et al.*, 1981).
2. HCMV is the world's leading cause of viral congenital infections and because of late-onset and often long-term neurological complications is a very costly disease in terms of therapy and patient care. In addition, as stated above, it can be vertically transmitted even in an immune mother, which lends importance to the determination of mechanism.
3. Detection assays, reagents, various strains of virus and expertise in dealing with HCMV are readily available at the University of Alberta.

2.2.2 Trophoblast Culture Model

Various models exist to look at the interactions of HCMV within the placenta. The most relevant but often impractical are direct studies of the pregnant woman. Since human manipulation is unethical, animal models have emerged. Studies in pregnant guinea pigs using a guinea pig specific CMV (GPCMV) have been particularly insightful in view of the similarity of placental architecture to humans and the similar ability of GPCMV to cross the placenta (reviewed in Bia *et al.*, 1983). While this provides an intact *in vivo* snapshot of the placenta during transmission, the ability to manipulate and study specific placental cells to pinpoint the mechanism of transmission is lacking. Perfusion of the whole or parts of the human placenta provide a picture of the intact organ but again studies are limited as described above along with the inability to maintain the tissue for the length of time required to study infection by this slow growing virus. Explant studies allow for the interaction of various placental cell types in context and provide a good model to study first trimester tissues, particularly those that have differentiated along the extravillous trophoblast pathway. The limitations of specific manipulation and decreased culture time also apply here. Studies performed on choriocarcinoma cells bypass these

limitations but the transformed and primarily extravillous character of these cells make results obtained difficult to extrapolate to chorionic villous trophoblasts. Highly purified CT stem cells isolated from chorionic villi of term or first trimester placentae and cultured in vitro under conditions that allow maturation are readily manipulated and offer a model to directly address the parameters of direct infection of these cells.

The villous trophoblast isolation and culture techniques developed by Morrish et al (Morrish, Siy, 1986) and modified in the Guilbert laboratory (Yui *et al.*, 1994) offer a culture model best suited for the study of infection by HCMV for the following reasons:

1. The isolated trophoblasts are highly purified (>99.99%, Kilani *et al.*, 1997) which reduces the possibility of HCMV infection in susceptible fibroblasts that could complicate results. Trophoblast cultures remain predominantly mononuclear, thus resembling immature CTs at one day of culture and are referred to as –EGF cultures throughout this thesis. Spontaneous differentiation with respect to expression of various hormones and proteins occurs in these cultures over time (Yui *et al.*, 1994; Morrish *et al.*, 1997).
2. Addition of epidermal growth factor (EGF) to isolated trophoblast cultures for five days will accelerate differentiation with respect to hormone production and will also induce extensive syncytialization (Morrish *et al.*, 1987; Yui *et al.*, 1994). These cultures then resemble the mature ST and are referred to as +EGF cultures throughout this thesis.
3. The viability of each of these cultures can be maintained for greater than three weeks. Isolated trophoblasts do not proliferate (Contractor, Sooranna, 1982; Kliman *et al.*, 1986; Aplin, 1991; Yui *et al.*, 1994; Garcia-Lloret *et al.*, 1996) and since HCMV replicates more rapidly in proliferating than quiescent cells (Stinski, 1977), extended culture time may be essential for detection of permissive infection in these cells.

2.3 EXPERIMENTAL APPROACH

My first objective was to determine if cultured trophoblasts isolated from first trimester and term placentas could be infected with various strains of HCMV and if so to follow the progression of infection. Permissive infection was evaluated by detection of

HCMV-specific immediate early (IE) and early proteins by immunohistochemistry and by detection of progeny virus. These initial studies are presented in Chapter 4.0.

Although permissive HCMV infection was detected in cultured trophoblasts, the infection frequency was very low. Since any further investigation into the effects of viral infection on barrier function of trophoblasts would require a higher infection frequency to be meaningful, my next objective was to optimize viral challenge protocols in trophoblast cultures. These studies led to the observations of differential susceptibility, progression and cellular outcome to HCMV infection in –EGF and +EGF trophoblast cultures that were investigated further (Chapters 5.0 and 6.0).

Infection and progeny virus levels in cultured trophoblasts were now at levels sufficient for further study of barrier function. My third objective then was to determine if progeny virus from infected trophoblasts could be released basally (or towards the fetus *in vivo*). I first had to develop an effective barrier culture model that would allow access to both apical and basolateral trophoblast surfaces (Chapter 7.0). Finally, infected trophoblast barrier cultures could be assessed for directional release of progeny virus (Chapter 8.0).

CHAPTER 3.0 MATERIALS AND METHODS

3.1 GENERAL MATERIALS AND METHODS

3.1.1 Cells

3.1.1.1 Term Villous Cytotrophoblasts

Placentas were obtained after normal term delivery or elective cesarean section from uncomplicated pregnancies. Villous CTs (>99.99% pure) were isolated by trypsin/DNase digestion of minced chorionic tissue and immunoabsorption onto Ig-coated glass bead columns (Biotex, Edmonton, Alberta) as previously described (Yui *et al.*, 1994; Kilani *et al.*, 1997) using anti-CD9 (Clone 50H.19; Maclean *et al.*, 1982; Morrish *et al.*, 1991) anti-MHC class I (W6/32, Harlan Sera-Lab, Crawley Down, Sussex, England) and anti-MHC class II (Clone 7H3) antibodies for immunoelimination. The purified cells were routinely cryopreserved and after thawing were washed twice in Iscove's Modified Dulbecco's Medium (IMDM, GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, GIBCO). The cells were seeded at 10^5 per microwell in 100 µL 10% FBS/IMDM in 96-well tissue culture dishes (NUNC #167008, GIBCO), incubated for 4 hrs at 37°C in a 5% CO_2 humidified atmosphere, the nonadherent cells and debris removed with pre-warmed IMDM and the cells replenished with 10% FBS/IMDM and 50 µg/mL gentamicin. Trophoblasts were isolated from several different placentas for this study. All preparations contained fewer than five vimentin-positive cells (fibroblasts) per microwell after the 4 hr wash. Syncytialization of cultured CTs was induced by treatment with 10 ng/mL recombinant human EGF (Prepro-Tech, Rocky Hill, NJ) for five days (Morrish *et al.*, 1987; Garcia-Lloret *et al.*, 1996; Morrish *et al.*, 1997) and assessed by immunohistochemically staining fixed cells with anti-desmoplakin monoclonal antibody (Sigma Immunochemicals, St. Louis, MO) to visualize desmosome-containing tight junctions (Douglas, King, 1990) as previously described (Yui *et al.*, 1994).

3.1.1.2 First Trimester Villous Cytotrophoblasts

Placental chorionic tissue was separated microscopically from fetal material obtained from elective abortions performed at 10 to 15 weeks gestation. Chorionic cells

were isolated as described previously for term cells (Yui *et al.*, 1994) with the following modifications: 10 ml of tissue was harvested per placenta; trypsin-DNase digestion was performed at the same concentration for the same number of times as with term placentas, but with 1:1 volumes of tissue to trypsin-DNase at a reduced time of 5 mins. Cell purification was carried out on glass bead columns as described above. CT preparations from first trimester placentas used in this study contained less than 0.02% vimentin-positive cells. Culture and induction of syncytialization of first trimester villous CTs was performed as described for term CTs using a 3-day incubation with EGF rather than 5 days. Ethics approval was obtained for all studies performed on human tissue from the Health Research Ethics Board in conjunction with the University of Alberta, Capital Health and the Caritas Health Group (Issue #3675).

3.1.1.3 Placental Fibroblasts

Placental fibroblasts (PF) were isolated from first trimester chorionic cell suspensions prior to antibody treatment and column purification by plating the suspensions in 60 x 15 mm tissue culture dishes for 60 mins, followed by removal of nonadherent cells and culture in 10% FBS/IMDM. Adherent cells grown to confluency were lifted by treatment with 0.05% trypsin-EDTA (GIBCO), washed in 10% FBS/IMDM and further propagated in 100 x 20 mm tissue culture dishes. Confluent cultures were passaged at least five times to ensure >99% purity, as assessed by immunohistochemical staining for vimentin and cryopreserved in 10% dimethyl sulphoxide (DMSO; Fisher) in FBS. Before experimental use, PF were thawed and cultured in 10% FBS/IMDM and 50 μg/mL gentamicin until confluent and passaged at least once.

3.1.1.4 HEL Fibroblasts

Human embryonic lung fibroblasts (HEL) obtained from Dr. J. Preiksaitis (Department of Medicine) were propagated in Eagle's Minimum Essential Medium (MEM), supplemented with 10% FBS and 50 μg/mL gentamicin. For HCMV infection assays, the cells were plated in 10% FBS/MEM at a concentration of 4×10^4 per 100 μL in 96-well tissue culture plates. All experiments were carried out on confluent cultures with media changes every 48 hrs.

3.1.2 Immunohistochemical Staining

Infected and uninfected cultures were washed twice with phosphate buffered saline (PBS), fixed in ice-cold methanol for 10 min at -20°C and washed three times with phosphate-buffered saline (PBS). Endogenous peroxidase activity was neutralized by a 30-min incubation at RT with 3% H_2O_2 and followed by a 1 hr incubation at RT in 10% non-immune goat serum (Zymed/Intermedico, Markham, CA) to block nonspecific sites. Primary antibodies detecting either HCMV IE (detecting p72, Specialty Diagnostics, Dupont), or HCMV pp65 (detecting pp64/pp65, Biotest, Germany) antigens, and their respective isotype controls, IgG_{2a} (Zymed/Intermedico) and IgG_1 (Dako Corporation, Carpinteria, CA) were added, the plates sealed with parafilm and incubated overnight at 4°C. After thorough washing with PBS, secondary antibody (biotinylated goat anti-mouse IgG) and streptavidin-peroxidase conjugate (Streptavidin Biotin System, Histostain-SP Kit, Zymed) were added according to the manufacturer's instructions. Following a PBS wash, either Ni-DAB substrate (95 mg diaminobenzidine, 1.6 g NaCl, 0.136 g imidazole, 2 g $NiSO_4$, made up to 200 ml with 0.1 M acetate buffer, pH 6.0) yielding a dark brown precipitate or amino-ethyl carbazole (AEC) yielding a red precipitate was added for 2 to 5 mins. The plates were then washed with ddH_20. The frequency of IE or pp65 positive nuclei and infection foci were determined at all time points. The number of nuclei per infection foci ranged from one in -EGF cultures within a week of infection to as high as 50 for +EGF cultures at 20 days of culture. In some cases doublestaining by incubation with a second primary antibody, either desmoplakin (Des; ICN ImmunoBiologicals, Costa Mesa, CA), or vimentin (Vim, Clone V9, Dako Corporation), was carried out immediately and the secondary antibody and streptavidin-peroxidase conjugate steps repeated as above, using Ni-DAB for the first substrate and AEC for the second. All cultures were counterstained with hematoxylin and photographs taken within a week.

3.1.3 Statistics

All statistics carried out are described in the respective figure or table legends. Most data presented in this thesis are single representative experiments of multiple experiments performed. The high variability between placental preparations is such that

combining data from multiple experiments was largely not possible. Additionally, due to the type of outcome variable measurement (counted versus continuous), only non-parametric tests were valid to analyze most of the data. Thus although each experiment showed the same trend, performing statistics on combined experiments was not possible. Since paired T tests are parametric analyses and require multiple independent experiments to be useful, they were not relevant to use. All experiments analyzed were done with the help of Damon Mayes, a biostatistician at the Perinatal Clinical Research Center, University of Alberta.

3.2 MATERIALS AND METHODS SPECIFIC FOR CHAPTER 4.0

3.2.1 HCMV

3.2.1.1 Virus Stock Preparations

HCMV laboratory strains, AD169, Davis, Towne and a clinical isolate from a congenitally infected infant were obtained from Dr. J. Preiksaitis (Department of Medicine) and were passaged on confluent HEL cells in 2% FBS/MEM at a multiplicity of infection (MOI) of 0.05 which is equivalent to 1 virus for every 20 cells. When 80 to 90% of the cells demonstrated cytopathic effects, the cultures were frozen and thawed three times to release infectious virus. Supernatants and cell lysates were passed through 0.45 μm filters (MILLEX-HV, Millipore Products Division, Bedford, MA) and stored in liquid nitrogen until use. Viral titers were determined by centrifugal enhancement (Osborn, Walker, 1968) of serial titrations on confluent HEL fibroblasts in 96-well plates. The plates were centrifuged for 45 mins at 2500 rpm in a GCL-2 Sorvall centrifuge, the wells washed five times with warm MEM and the plates incubated for a further 18 hrs in fresh 2% FBS/MEM. The cultures were fixed in ice-cold methanol and immunohistochemically stained for HCMV IE antigen as described above. Each IE-positive nucleus was equated to one plaque-forming unit (PFU) of infectious virus and the titer of virus was determined within a linear dose-response concentration range as PFU/mL.

3.2.1.2 Viral Challenge Protocols

Viral challenge with each HCMV strain or isolate at various MOIs was carried out in serum-free IMDM for 2 hrs at 37°C in 5% CO_2. The number of cells per well was established at all culture times as described in Section 3.2.2.2. The trophoblast cultures were challenged as follows: +EGF term trophoblasts 5 days after plating, -EGF term trophoblasts 1 day after plating and +EGF first trimester trophoblasts 3 days after plating. The cultures were then washed five times with serum-free IMDM, incubated in fresh 2% FBS/IMDM with or without EGF for various times post-infection and the media changed every 96 hrs. Viral challenge of PF and HEL cultures were carried out as described above in serum-free MEM, followed by incubation in 2% FBS/MEM. All placental preparations were tested for initial or reactivated HCMV infection by including uninfected control cultures stained for IE and pp65 antigens.

3.2.1.3 Assessment of Infection in Trophoblasts

Infection in trophoblast cultures was assessed in two ways: first, total IE-positive or pp65-positive nuclei per well were counted and divided by the total nuclei per well as estimated by DNA content (Section 3.2.2) to give percent positive nuclei per well. Since most cells in –EGF cultures are mononucleated, this value was equated to percent positive cells per well. In this chapter, two assumptions were made with respect to +EGF cultures: 1) All nuclei in an infected multinucleated cell are IE-positive (e.g. Figure 4.1A) and 2) All cells in +EGF cultures infect with equal susceptibility whether multinucleated or not. If these assumptions are true, both the numerator and denominator of the percent positive nuclei value can be divided by 4, the average number of nuclei per cell in +EGF cultures (Kilani *et al.*, 1997). The percent positive cell value obtained is thus identical to the percent positive nuclei value.

The second assessment method was to count foci of infection per well. Foci of IE or pp65-positive nuclei consisted of one or more closely associated positive nuclei. Early in infection IE-positive foci likely represent single cells, whether mononucleated or multinucleated. Later in infection, foci likely represent multiple cells. While IE staining was consistently strong, pp65 staining tended to be differential. Strongly pp65-positive

nuclei were often surrounded by weakly stained nuclei (Figure 4.1B). When foci were counted, the strongly stained nuclei were each assessed as one foci.

3.2.1.4 Infectious Virus Titers in Supernatants or Cell Lysates

Supernatants were removed from cultures at various times post-challenge and frozen at -80°C until assessed for viral titer. Adherent cells were washed three times with PBS and lysed in 100 µL 2% FBS/IMDM by freezing and thawing three times (cell lysate). Viral titers in culture supernatants or cell lysates were assayed on HEL cultures as described in 3.2.1.1 and calculated as PFU/mL. Using these viral challenge protocols, no residual inoculum in the supernatant was detected 24 hrs after challenge (see Figure 4.5C).

3.2.2 Estimation of Cell Numbers in Culture

3.2.2.1 DNA Assay

The assay was a modification of a method previously described (Cesarone *et al.*, 1979). Cells cultured in 96-well plates were washed twice with PBS, 100 µL of double distilled water (ddH_2O) was added to each well and the plates frozen and thawed three times to lyse the cells. The samples were transferred to 96-well V-bottom plates (Nunc), mixed with equal volumes of Hoechst Dye solution [1 µg/mL Hoechst 33258 (Sigma Chemical, St. Louis, MO), 10mM Tris, 1mM EDTA, 2.1 M NaCl, pH7.4] and the fluorescence measured on an LS-5 Luminescence Spectrometer (Perkin Elmer, Norwalk, CT) using calf thymus DNA as a standard to calculate the amount of DNA per well in ng/mL.

3.2.2.2 Determination of Cell Numbers in Culture

The number of trophoblasts and fibroblasts in each experiment was determined by calculating the DNA content of parallel cultures using the DNA content for a human diploid nucleus of 6 pg. Calculations of fibroblast and –EGF trophoblast numbers were based on one nuclei per cell while +EGF trophoblast numbers were based on an average of four per cell (Xiao *et al.*, 1997; Kilani *et al.*, 1997). After virus challenge and washing (see above), the microwell cultures of trophoblasts contained from 4000 to 16,000 cells

per well, depending on the adherence properties of individual preparations, the time of culture (most preparations lose 20%-50% of their DNA content over a one-month culture period) and the multinucleated state of the culture.

3.3 MATERIALS AND METHODS SPECIFIC TO CHAPTER 5.0

3.3.1 HCMV

3.3.1.1 Optimized Viral Stock Preparation

Infected HEL cells were removed from the flask with a cell scraper (Becton Dickinson; Bedford, MA) and transferred to a 50 mL tube on ice. The cells and culture supernatant were sonicated using a Vibracell sonicator (Sonics and Materials Inc., Danbury, CT, USA) and the resulting lysate clarified by passage through a 0.45 μm filter as previously described (Section 3.2.1.1).

3.3.1.2 Optimized HCMV Viral Challenge Protocol

Viral challenge protocols for trophoblasts were optimized in this chapter with the following protocol adopted for all subsequent chapters: challenge was carried out in 2% FBS/IMDM for 24 hrs at 37°C in 5% CO_2 in the presence (+EGF cultures) or absence (-EGF cultures) of EGF. Any deviations from this protocol are noted in the individual figure legends. Media was changed every 48 hrs rather than 96 hrs as in Chapter 4.0. The number of cells per well was established at all culture times as described below.

3.3.1.3 Low pH and IVIG Treatments

Trophoblast cultures were challenged with HCMV for 24 hrs, washed with serum-free IMDM and incubated with a saline solution at a pH of 3.0 for 2 mins (Compton, 2000). The cultures were washed well, resuspended in 2% FBS/IMDM and further incubated according to individual figure legends. This low pH treatment was also performed on all infected cultures just prior to collection of cell lysates by three freeze-thaw cycles. Some cultures were challenged and washed as described above and incubated with intravenous immune globulin (IVIG), which contains neutralizing antibodies to HCMV (Tenold, 1983; Zaia, 1993), at a concentration of 10 mg/mL for 48 hrs prior to assessment of progeny virus in cell lysates and supernatants. This is the

lowest concentration of IVIG that gives >95% inhibition of infection on HEL fibroblasts after a 10-min preincubation (Figure 3.1).

3.3.1.4 Assessment of Infection in Trophoblasts

For this and all following chapters, infection in trophoblast cultures was assessed and reported as percent positive nuclei per well regardless of culture treatment. The total number of nuclei per well was determined as described in the next section.

3.3.2 Estimation of Cell Numbers in Culture

For this and all following chapters, the total number of nuclei per well in all cultures was calculated by multiplying the average number of nuclei counted in five fields by the total number of fields per well (123 at a magnification of 200X). MOI for each culture was determined using the total nuclei per well regardless of the state of nucleation.

3.3.3 MTT Assay

The assay is based on the method of Mosmann (Mosmann, 1983) where metabolic activity of the culture is detected by reduction of a yellow chromophore to blue and measured by absorbancy at 570 nm. Supernatants were removed from the cultures, replaced with 100 µL of a 0.5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) in IMDM plus 2% FBS. After a 4-hr incubation period, 150 µL of 0.04 M HCl in isopropanol was added to each well. The plate was wrapped in parafilm and left overnight in the dark at RT to dissolve the crystals. The optical density was determined the following day at 570 nm with a reference wavelength of 650 nm on a microplate reader (Molecular Devices, Menlo Park, CA).

3.3.4 Immunohistochemical Staining

Infected and uninfected +EGF and –EGF trophoblast cultures were washed twice with PBS, fixed in 4% paraformaldehyde buffered to pH 7.4 - 7.6 at RT for 10 mins and then washed three times with PBS. Cultures were immunohistochemically stained with an

antibody detecting placental alkaline phosphatase (PLAP; Sigma Immunochemicals) or its isotype control IgG_{2a} using the staining methods previously described (3.1.2). In some cases cultures were double stained for IE antigen using Ni-DAB substrate and PLAP using AEC substrate. No counterstaining was performed in order to best visualize small differences observed in PLAP staining compared to controls.

3.4 MATERIALS AND METHODS SPECIFIC TO CHAPTER 6.0

3.4.1 TUNEL Assay

The details of the TUNEL (Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling) assay have been described elsewhere (Gavrieli *et al.*, 1992; Yui *et al.*, 1994). Briefly, trophoblasts were fixed with acetone:methanol (1:1), then subjected to TUNEL for 60 mins at 37°C in a mixture of bio-16-dUTP (Sigma B3029) at 16.5 µM, dATP (Boehringer Mannheim, Laval Quebec) at 16.5 µM and TdT enzyme at 5 U/µL (Boehringer Mannheim) in TdT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, and 1 mM cobalt chloride). The reaction was terminated by adding 2X SSC (300 mM sodium chloride plus 30 mM sodium citrate). After washing the cells in ddH_2O, endogenous peroxidase activity was quenched in a 3% H_2O_2 solution and non-specific binding was reduced by incubation with blocking buffer (3% skim milk powder in PBS containing 0.5% Tween-20). The cells were incubated for 30 mins at RT with ExtrAvidin peroxidase (Sigma) diluted 1:75 in blocking buffer, washed 3 times with ddH_2O, then stained with AEC at RT until development of a red color (approximately three mins).

3.5 MATERIALS AND METHODS SPECIFIC TO CHAPTERS 7.0 AND 8.0

3.5.1 Cells

3.5.1.1 Culture of Cytotrophoblasts on Semi-Permeable Insert Membranes

Insert membranes (6.4 mm) precoated with fibronectin (Biocoat; Becton Dickinson) were soaked in 10% FBS/IMDM and 50 µg/mL gentamicin for 1 hr prior to cell plating. Trophoblasts were thawed, washed once and 2 x 10^5 cells in 10% FBS/IMDM added to each insert. The inserts were incubated at 37°C in a 5% CO_2 humidified atmosphere in specialized Falcon companion 24-well tissue culture plates (see

Figure 8.1; Becton Dickinson). Non-adherent cells were removed 4 hrs later by three gentle shaking washes and the cultures replenished with 200 µL per insert to 800 µL per culture well of 10% FBS/IMDM containing rHu EGF (10 ng/mL) to promote syncytialization. On the third and seventh days of culture, freshly thawed and washed trophoblasts from the same placental preparation were added as described above. The medium was changed in both insert and lower chambers every two days. Since HCMV does not readily diffuse through 0.45 µm pores, insert membranes with 3.0 µm pores, which allow HCMV diffusion, were consistently employed.

3.5.1.2 Madin-Darby Canine Kidney II Epithelial Cells (MDCK II)

MDCK II cells, a gift from Dr. Bruce Stevenson (Department of Anatomy and Cell Biology, University of Alberta), were propagated in Dulbecco's Modified Essential Medium (DMEM) supplemented with 5% FBS, 20 mM Hepes, 4.5 g/L glucose and 50 µg/mL gentamicin. Cells were lifted with 0.25% trypsin in 0.02% EDTA and plated onto fibronectin-coated 6.4 mm insert membranes with 3.0 µm pores. All experiments were performed on confluent cultures.

3.5.2 HCMV

3.5.2.1 Transepithelial Diffusion of Human Cytomegalovirus (HCMV)

4×10^5 PFU of HCMV strain AD169 were added to either cell-free or triple-seeded trophoblast inserts and the inserts placed in wells from 24-well Falcon companion tissue culture plates containing confluent HEL fibroblasts. After a 2-hr incubation the inserts were removed, the fibroblasts cultured an additional 18 hours in the 24-well plates and then immunohistochemically stained for IE antigen. IE-positive fibroblasts were each counted as one PFU and percent diffusion calculated from the original PFU of virus added to the insert.

3.5.2.2 Viral Challenge of Insert Cultures

Insert cultures were challenged with HCMV strain AD169 at an MOI of 10 after triple-seeded cultures achieved high TER and low ^{14}C-dextran diffusion. After 24 hrs of challenge the inside and outside of the culture inserts were treated with a low pH solution to remove adherent virus and carefully washed. Progeny virus was monitored over time

in three compartments: the supernatant of the culture insert (apical), the cell lysate (cells) and the supernatant of the culture well (basal; see Figure 8.1 for culture model diagram). To monitor for progeny virus, at various times after viral challenge each insert was carefully washed both inside and outside and placed into a well containing confluent HEL fibroblasts. After 24 hrs, the insert was removed and the HEL culture incubated a further 18 hrs before fixing and staining for IE antigen. Insert culture supernatants and cell lysates, prepared as previously described (3.2.1.3), were stored at -70°C and assayed upon completion of the experiment by titer on HEL fibroblasts. In a second series of experiments viral challenge was carried out after the first seeding of trophoblasts on the culture insert. After a low pH treatment to remove any externally adherent virus and extensively washing both inside and outside of the insert, the cells were cultured for a further 24 hrs. A second seeding of trophoblasts was then applied followed three days later by a third seeding and monitoring of progeny virus as described above commenced upon demonstration of high TER and low ^{14}C-dextran diffusion.

3.5.3 Immunohistochemical Staining of Insert Membranes

Insert cultures were stained as previously described (Section 3.1.2). In some cases insert cultures were counterstained with hematoxylin (Sigma) to visualize nuclei and Stat Stain (VWR, Mississauga, Ontario) to visualize cytoplasm. Once dry, the membranes were cut out of the insert and mounted in GVA Mounting Media (Zymed) on glass slides. Photographs were taken within a week of mounting.

3.5.4 Transepithelial Electrical Resistance (TER)

TER was measured with an Endohm tissue resistance measurement chamber (World Precision Instruments, Inc.; Sarasota, FL), consisting of a bottom chamber and cap containing apposing circular disc electrodes. All inserts were rinsed with PBS, allowed to equilibrate to RT in PBS and placed in the bottom chamber containing PBS. The Endohm cap was placed inside the insert, centering it on the bottom chamber electrode and the electrical resistance was monitored on a Millicell-ERS meter (Millipore/Continental Water Systems, Bedford, MA). To obtain values independent of the membrane area, TER measurements were multiplied by the effective membrane

diameter (0.3 cm^2) and reported as ohms x cm^2. All TER measurements are reported as the TER less the average TER of two cell-free inserts (22.8 ohms x cm^2).

3.5.5 Transepithelial Diffusion of Radiolabelled Compounds

Two compounds were used: ^{3}H-inulin (mw 5000; NEN, Boston, MA) and ^{14}C-methylated dextran (mw 2,000,000; Sigma Radiochemical, St. Louis, MO). The amounts added to each insert (maximum variance of 25% between experiments) were normalized to 14.3 pmole (50,000 dpm) of ^{3}H-inulin or 12.4 pmole (85,000 dpm) of ^{14}C-dextran in 200 µL. The inserts were placed in wells containing 800 µL of fresh medium, removed at each time point and placed in wells with fresh medium until the next time point. 400 µL aliquots of the bottom wells at each time point were mixed with scintillation fluid (Opti-Fluor, Canberra Packard, Ontario, Canada), counted in a scintillation counter and the normalized cumulative transepithelial diffusion reported in pmoles as a function of time for each radiolabelled compound. Initial transepithelial flux was calculated as the diffusion velocity in the first 15 mins normalized to the surface area of the membrane and expressed as pmoles/hr/cm^2. 100-fold excess unlabelled (cold) dextran (mw 2,000,000; Sigma) was added with ^{14}C-dextran in some experiments to a final volume of 200 µL per insert. Fresh inserts placed in culture medium 24 hrs prior to each diffusion assay were used as cell-free insert controls.

3.5.6 Electron Microscopy

All EM procedures were carried out by Dr. R. Sherburne (Department of Medical Microbiology and Immunology).

3.5.6.1 Transmission Electron Microscopy (TEM)

Insert membranes were fixed with freshly prepared 2.5% (v/v) glutaraldehyde in phosphate buffer pH 7.3 for one hr, washed three times in phosphate buffer and further fixed in 1% (w/v) osmium tetroxide overnight at 4°C. After washing in phosphate buffer, the insert was dehydrated in a graded series of ethanol at RT and the membrane cut out of the inserts and transferred to propylene oxide twice for 30 mins. The membranes were transferred to a 1:1 mixture of propylene oxide and LX 112 resin (Ladd Research

Industries, Burlington, Vt.) and left uncapped for 24 hrs at RT, then transferred to pure LX 112 and cured for 24 hrs at 60°C. Sections cut on a Reichert-Jung Ultracut were placed on a 3 mm 200 mesh formvar-coated copper grid and stained with 5% uranyl acetate for 20 mins and lead citrate for 6 mins. Images were recorded on Kodak #4489 electron microscope film using a Philips model 410 transmission electron microscope. For analysis of cell layers, ten ultra-thin sections were cut from six areas, three from each side of an insert (50 µm apart) with 12-day triple-seeded trophoblasts, to obtain representative samples of the membrane. Sections were placed on super high transmission 200 mesh hexagonal copper grids (Pointe-Claire, Quebec, Canada) and analysis of 437 fields performed at 9100x magnification.

3.5.6.2 Scanning Electron Microscopy (SEM)

Samples for scanning electron microscopy were processed as for TEM up to the 100% ethanol step and then critical point dried (Balzers Critical point dryer #CPD 030). The membranes were carefully cut out of the insert, mounted on standard Cambridge scanning electron microscopy stubs and examined in a Hitachi S 4000 field emission scanning electron microscope at an accelerating potential of 5.0 kV.

3.5.7 Detection of MMP-9 by Gelatin Zymography.

Continuous triple-seeded trophoblast cultures on 3.0 µm insert membranes were gently washed with warm IMDM and then refed with 37°C IMDM. In order to limit apical (upper chamber) to basal (lower chamber) diffusion of secreted proteins, the fluid level of the lower chamber was maintained 5 mm higher than that of the upper chamber. After a two-hour incubation at 37°C, the activity of MMP-9 in both chamber supernatants were measured by zymography under non-reducing conditions as previously described (Sawicki *et al.*, 1997). Briefly, 20 µg of protein from conditioned media was electrophoretically separated on 8% SDS polyacrylamide gels (SDS-PAGE) with copolymerized gelatin (2 mg/mL) as a gelatinase substrate. The gelatinolytic activity was detected as a sharp 92 kDa (proMMP-9) band of cleared gelatin which was quantified by measuring the density of the cleared bands using a ScanJet 3c scanner and SigmaGel measurement software. Activities were expressed as arbitrary absorbance units and

normalized to reflect the total secretion into each chamber. All zymography was carried out by Dr. G. Sawicki (Department of Pharmacology).

3.5.8 Confocal Microscopy

Triple-seeded trophoblast insert cultures prepared as described in Section 3.5.1.1 were challenged with HCMV AD169 at an MOI of 10 for 24 hrs and cultured for a further 11 days with medium changes every 48 hrs. The cultures were fixed in ice-cold methanol for 10 mins at -20°C and washed well with PBS. A primary antibody detecting HCMV pp65 antigen (Biotest, Germany) was added and the inserts incubated overnight at 4°C. After washing extensively, a fluorescenated secondary antibody (goat α mouse; Alexa Fluor 546, Molecular Probes) was added for 30 mins. After washing, DAPI nucleic acid stain (Molecular Probes) was added for 10 mins to counterstain all nuclei, the inserts were again washed well and mounted on glass coverslips. Confocal analysis was done using a Zeiss microscope with an F-Fluar 40x objective lens and analyzed with the LSM510 software program. Using the krypton-argon laser, the inserts were scanned for the Alexa Fluor 546 (red). DAPI (emission at 461) was detected using the UV laser (originally blue but converted to green by the software program). The insert surface (green) in Figure 8.3A was also visualized using the UV laser. The invaluable help of Dr. Xue-Jun Sun in the Cell Imaging Facility at the Cross Cancer Institute is gratefully acknowledged.

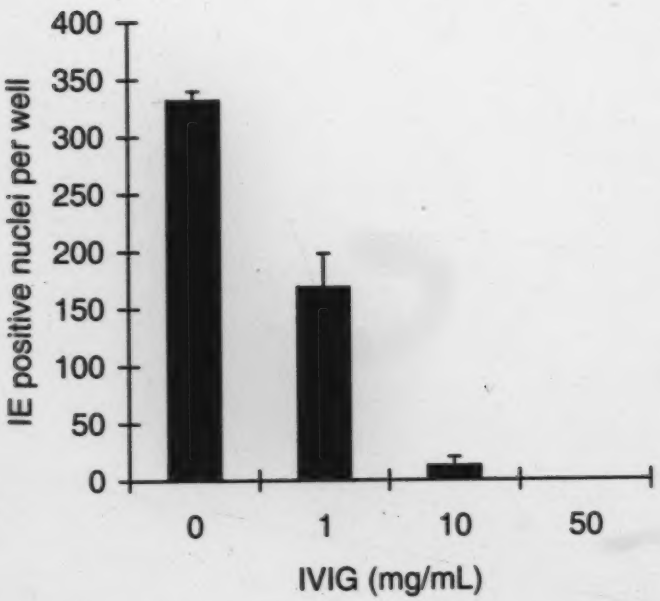


Figure 3.1 Inhibition of HCMV infection on trophoblasts by IVIG pretreatment. Stock HCMV virus was thawed and divided into 5 aliquots, each equivalent to 0.01 MOI on HEL fibroblasts (volume less than 5 µl per aliquot). Each aliquot was added to 100 µl of 2% FBS/IMDM containing various concentrations of IVIG: 0, 1, 10 or 50 mg/mL and incubated at 37°C for 10 mins. HEL fibroblasts were challenged with each of these solutions for 2 hrs., washed and further incubated for 18 hrs. Assessment of infection was done by staining for IE antigen. Results are presented as the average ± SD of triplicate wells from one of two independent experiments performed.

CHAPTER 4.0 PERMISSIVE CYTOMEGALOVIRUS INFECTION OF PRIMARY VILLOUS TERM AND FIRST TRIMESTER TROPHOBLASTS

(The majority of data presented in this chapter was published in 1998 in *Placenta* 72(6):4970-4979 in an article entitled "Permissive Cytomegalovirus Infection of Primary Villous Term and First Trimester Trophoblasts," by D.G. Hemmings, R. Kilani, C. Nykiforuk, J. Preiksaitis and L.J. Guilbert).

4.1 INTRODUCTION

The role of ST in transmission of HCMV across the placental barrier is unclear. Results from *in vivo* studies are difficult to assess because placentas obtained from stillbirths, symptomatic congenitally infected infants or those with chronic villitis tend to be preferentially studied (Quan, Strauss, 1962; Hayes, Gibas, 1971; Monif, Dische, 1972; Benirschke *et al.*, 1974; Mostoufi-Zadeh *et al.*, 1984; Garcia *et al.*, 1989; Schwartz *et al.*, 1992; Muhlemann *et al.*, 1992; Sinzger *et al.*, 1993). Such third trimester placentas, and the trophoblast in particular, rarely display the inclusion bodies characteristic of permissive HCMV infections (Benirschke *et al.*, 1974; Mostoufi-Zadeh *et al.*, 1984; Garcia *et al.*, 1989; Muhlemann *et al.*, 1992; Sinzger *et al.*, 1993; Nakamura *et al.*, 1994). Immunohistochemical analysis of sections from third trimester placentas displaying chronic villitis reveal IE (Muhlemann *et al.*, 1992; Sinzger *et al.*, 1993; Schneeberger *et al.*, 1994) but not early nuclear (Muhlemann *et al.*, 1992; Schneeberger *et al.*, 1994) or late (p150; Sinzger *et al.*, 1993) antigens in trophoblasts, suggesting abortive infections (Sinzger *et al.*, 1993). *In situ* hybridization detects HCMV DNA primarily in stromal cells and occasionally in the trophoblast of term placentas with chronic villitis (Sachdev *et al.*, 1990). Term placentas perfused *in vitro* and challenged with high titers of a HCMV laboratory strain for up to 9.5 hours are non-permissive within this short experimental time frame (Muhlemann *et al.*, 1995).

In contrast, placentas from first or second trimester abortions contain nuclear inclusions frequently in stromal cells (Blanc, 1961; Altshuler, McAdams, 1971; Monif, Dische, 1972; Benirschke *et al.*, 1974; Schwartz *et al.*, 1992) and more rarely in trophoblasts (Garcia *et al.*, 1989; Sinzger *et al.*, 1993) with some expression of pp65

antigen in the trophoblast (van Lijnschoten *et al.*, 1994) indicating a permissive trophoblast infection during the first half of gestation is possible. *In vitro* infections of first trimester placental explants show permissive infections of the trophoblast by morphological and immunohistochemical criteria and detection of viral DNA (Amirhessami-Aghili *et al.*, 1987; Amirhessami-Aghili *et al.*, 1989). In guinea pigs, detection of intranuclear inclusions and expression of GPCMV antigens in ST at all stages of gestation indicate permissive infections in trophoblasts occur in this animal model (Griffith *et al.*, 1985). Highly purified term trophoblasts express IE antigens after HCMV challenge but do not release virus into culture supernatants unless co-infected with either HIV-1 (Toth *et al.*, 1995b) or HTLV-1 (Toth *et al.*, 1995a). These results are compatible with the *in vivo* findings of infrequent non-permissive trophoblast infections at term. However, the 40% transmission rate resulting from primary maternal infections and the more frequent indications of permissive infections in first trimester trophoblasts are difficult to reconcile on the basis of such co-infections. Frequent stromal infections and evidence of focal trophoblast infections suggest a route of transmission by infection of ST should be investigated.

Although apparently straightforward, the development of an effective culture model of ST infection by HCMV must address two interdependent problems: fibroblast contamination and long term culture viability. Placental fibroblasts are likely preferred targets for this virus not only because laboratory strains are passaged in fibroblasts but also because placental fibroblasts, unlike primary villous trophoblasts, proliferate in culture (Contractor, Sooranna, 1982; Kliman *et al.*, 1986; Aplin, 1991; Yui *et al.*, 1994; Garcia-Lloret *et al.*, 1996). HCMV replicates more readily in proliferating than quiescent cells (Stinski, 1977; DeMarchi, Kaplan, 1977; Tanaka *et al.*, 1985) and would be predicted to replicate more slowly in trophoblasts than fibroblasts. Permissive infection by HCMV also requires viable (healthy) cultures (Mocarski, 1996). Demonstration of delayed infection kinetics in macrophages (Fish *et al.*, 1995), a rhabdomyosarcoma cell line (Cinatl *et al.*, 1994), primary neurons (Poland *et al.*, 1994) and a colon epithelial cell line (Jarvis *et al.*, 1999) reinforces the possibility of slow virus replication and the necessity for long-term viable trophoblast cultures. However, primary trophoblasts have rarely been cultured for longer than seven days because of fibroblast overgrowth or loss

of viability (Kliman *et al.*, 1986; Douglas, King, 1990; Yui *et al.*, 1994; Fazely *et al.*, 1995).

A culture model of highly purified (>99.99%) term trophoblasts that maintain viability for longer than 3 weeks in culture has been developed in the Guilbert laboratory (Kilani *et al.*, 1997) and modified to obtain highly purified first trimester trophoblasts. These primary trophoblasts can be induced to undergo syncytialization and express high levels of human chorionic gonadotropin (hCG), human placental lactogen (hPL) and placental alkaline phosphatase (PLAP) when cultured in the presence of EGF for 5 days (+EGF cultures) and thus resemble the more differentiated ST (Morrish *et al.*, 1987; Yui *et al.*, 1994). Trophoblasts cultured without EGF for one day (-EGF cultures) remain predominantly mononuclear, resembling immature CT, and show variable expression of the above markers that spontaneously increase with time (Yui *et al.*, 1994; Morrish *et al.*, 1997).

This chapter describes results obtained in the initial investigation of my first objective: can cultured trophoblasts be permissively infected with HCMV? Using the standard viral challenge protocol developed in cultured fibroblasts, I demonstrate that trophoblasts isolated from term and first trimester placentas can be permissively infected with HCMV. The infection is inefficient in both +EGF and –EGF cultures even with high viral inoculum, proceeds slowly and progeny virus remains predominantly cell associated.

4.2 RESULTS

4.2.1 Term villous trophoblasts could be infected with cell-free HCMV

Primary villous trophoblasts cultured with EGF (+EGF cultures) form within five days a predominantly multinuclear cell layer (ST-like, Figure 4.1) whereas cells cultured without EGF (-EGF cultures) form a predominantly mononuclear cell layer (CT-like; (Yui *et al.*, 1994)). Viral challenge of +EGF trophoblasts at day 5 of culture with HCMV strain AD169 for two hours followed by extensive washing and further culture resulted in expression of both HCMV IE antigen and pp65, a viral matrix protein expressed at early to late infection times, 12 days after challenge (Figure 4.1A, 4.1B). Each multinucleated (syncytialized) cell, demarcated by desmoplakin staining, was generally IE positive in all nuclei or none (e.g., Figure 4.1A). The solid black arrows point to evidence of overlapping cells in these cultures. In Figure 4.1A a single IE-negative nucleus is lying underneath an IE-positive multinucleated cell and in 4.1C the arrow points to a nucleus clearly lying beneath a desmoplakin-positive cell boundary.

To determine the kinetics of infection, +EGF cultures were challenged with AD169 and the percentage of IE and pp65 positive cells determined at various times after challenge (see Materials and Methods Specific to Chapter 4.0 for the method to estimate positive cells in this predominantly multinucleated culture). The numbers of IE and pp65 positive cells increased continuously throughout the 21-day culture period (Figure 4.2A). However, the increase of IE positive cells was observed earlier and IE-positive cells were consistently more numerous than pp65-positive cells. The data shown in Figure 4.2A is one of seven independent experiments carried out on five different placental trophoblast preparations. Between 18 and 21 days after viral challenge at an MOI of 1.0, the maximum fraction of IE-positive cells never exceeded 15% and <3% were positive for pp65 antigen using these challenge protocols.

Although the total number of IE-positive nuclei per well consistently increased with time, the number of infected foci (containing one or more IE-positive nuclei) did not, suggesting that the infection progresses laterally from cell to cell (Figure 4.2B).

4.2.2 Infected cells in culture were predominantly trophoblasts

Placental fibroblasts, common contaminants of primary trophoblast cultures (Kliman *et al.*, 1986), could be infected with AD169 almost as efficiently as human embryonic lung (HEL) fibroblasts. At an MOI of 0.1, 1.6 ± 0.17 % IE-positive nuclei were detected four days after viral challenge compared to 1.5 ± 0.36 % in HEL fibroblasts challenged at an MOI of 0.005. It was therefore possible that the rather low frequency of infection observed in term +EGF cultures could be attributed to contaminating fibroblasts. Fibroblasts, as well as other contaminating villous stromal cells such as macrophages and endothelial cells, can be immunohistochemically distinguished from trophoblasts by the former cells' expression of the intermediate filament protein vimentin. Analysis of the seven preparations of trophoblasts used in this chapter for vimentin-positive cells between 10 and 12 days after viral challenge showed 1.14 ± 1.17 positive cells in +EGF cultures and 1.08 ± 1.56 positive cells in -EGF cultures. Since there were between 4000 and 16,000 cells estimated in these cultures (see Methods Specific to Chapter 4.0), the average contamination frequency was between 0.03% and 0.007%. In an experiment using only one of these preparations (chosen for its unusually high number of vimentin-positive cells in the presence of EGF), the number of vimentin-positive cells did not exceed ten per microwell over a 20-day infection period (Figure 4.3A). Thus, it is unlikely that a significant fraction of the 15% IE-positive or the 2-3% pp65-positive cells observed three weeks after virus challenge are fibroblasts. Double staining of the cultures for IE antigen and vimentin 12 days after viral challenge confirmed this prediction: greater than 99% of IE-positive nuclei (in this experiment 495 of 496 counted in three wells) were vimentin-negative and thus trophoblasts (Figure 4.3B). Interestingly, most of the vimentin-positive cells were not IE positive (e.g., the vimentin-positive cell in Figure 4.3B is IE negative).

4.2.3 Infected trophoblasts produced infectious progeny virus that remained predominantly cell-associated

A permissive infection in trophoblasts was demonstrated by the presence of infectious progeny virus, titered on HEL fibroblasts, in culture supernatants (Figures 4.4 and 4.5C and D). However, exact times and extent of virus release into culture

supernatants varied between trophoblast preparations with some (e.g., Figure 4.6B) releasing no detectable virus. Differences in virus release were not due to fibroblast contamination since experiments in which there was appreciable release (a mean of 531 ± 450 PFU/ml) between days 8 and 20 after infection had microwells containing 1.03 ± 1.23 vimentin-positive cells, while those with very low release (a mean of 1.08 ± 1.48 PFU/ml) had 1.66 ± 1.65 vimentin-positive cells per microwell.

The variability and low titers of infectious virus released from infected trophoblasts (e.g., Figure 4.5C, 4.5D and 4.6B) suggested intracellular accumulation of virus, a phenomenon occurring in macrophages (Fish *et al.*, 1995). After washing infected cultures extensively, cell lysates were prepared and found to contain infectious virus, often at times when none were detected in culture supernatants (e.g., Figure 4.4, before day 16). In cultures where ratios of cell-associated to released virus could be calculated (e.g. those releasing detectable virus), greater than 100-fold more infectious virus was found in cell lysates than supernatants. This same ratio in infected HEL cultures was usually less than one (e.g., Table 6.3). In some preparations (e.g., Figure 4.6B), virtually all progeny virus remained cell-associated over the entire experimental period.

4.2.4 Susceptibility to and progression of HCMV infection was independent of trophoblast differentiation state

To determine whether the differentiation state of villous trophoblasts affected HCMV susceptibility and infection progression, IE-positive foci were monitored over time in trophoblasts from two different placental preparations cultured with and without EGF (Figures 4.5 and 4.6). Since all nuclei in an infected multinucleated cell are usually IE-positive (e.g. Figure 4.1A), each IE-positive focus likely represents a single infected cell, whether mononucleated or multinucleated, early in infection. Once cell-associated progeny virus is detected (Figure 4.4), each focus likely represents many cells infected in a cell-to-cell manner (Figure 4.2). Early in infection, the number of IE-positive foci or cells was very low and did not differ between +EGF and –EGF cultures (Days 1 and 4; Figure 4.5A and B) suggesting two possibilities: 1) the initial susceptibility to HCMV infection is not influenced by the differentiation state of the trophoblasts or 2) viral

challenge protocols are suboptimal and meaningful differences between cultures could not be assessed.

The differences in the number of infected foci found in +EGF and –EGF cultures during the first two weeks after a two-hour viral challenge were neither large nor reproducible between trophoblast preparations (e.g., Figures 4.5A, 4.5B and 4.6B). Any divergence between the numbers of infection foci in +EGF and -EGF cultures corresponded to the appearance of infectious virus in culture supernatants (day 8 in Figure 4.5C and 4.5D) but late release of infectious virus was not reproducible between preparations (e.g., Figure 4.6B).

Trophoblasts do not proliferate in vitro (Contractor, Sooranna, 1982; Kliman *et al.*, 1986; Aplin, 1991; Yui *et al.*, 1994; Garcia-Lloret *et al.*, 1996) and cultures lose from 20%-50% of their DNA content over a one-month period (Yui *et al.*, 1994). To confirm that differential cell loss from infected +EGF or –EGF cultures does not skew the above results, DNA content was measured in infected and mock infected cultures. Viral challenge of trophoblasts did not increase the normal loss of DNA either in the presence or absence of EGF over a three-week culture period (Figure 4.7).

4.2.5 Trophoblasts isolated from first trimester placentas could be permissively infected with HCMV

Although *in utero* transmission to the fetus following primary maternal infection can occur at any point during gestation, infection during the first trimester results in the most severe consequences for the fetus (Stagno *et al.*, 1986; Britt, Vugler, 1990; Boppana *et al.*, 1993). I therefore asked whether villous trophoblasts isolated from first trimester placentas could be permissively infected by HCMV, and if so, whether the kinetics and extent of infection differed from term trophoblasts. The expression of HCMV IE and pp65 antigens was determined between one and nine days after challenge with AD169 at an MOI of 1.0 (Figure 4.8A). Both antigens appeared more rapidly and were more frequent in first trimester (Figure 4.8A) than in term (Figure 4.2) trophoblast cultures. First trimester cultures were double-stained for vimentin and IE antigen (to detect infected fibroblasts). Out of 2560 IE-positive nuclei counted in four wells, only three were found in vimentin-positive cells, thus >99% of infected cells were trophoblasts. As

with pp65 expression, infectious virus production occurred earlier in first trimester (Figure 4.8B) than term cells (Figure 4.4). Although three days after viral challenge the ratio of cell-associated to supernatant virus was only 6, it increased to approximately 1000 by six and nine days after viral challenge (Figure 4.8B). Thus, progeny virus in first trimester trophoblasts, as with term cells, remained cell-associated, but more cells were infected and the infection progressed faster in first trimester compared to term cultures.

4.2.6 HCMV infection was less efficient in trophoblasts than in fibroblasts

The above infection experiments were carried out at a fixed MOI of HCMV for each cell type. To compare the initial interaction efficiency of virus with confluent HEL fibroblasts and +EGF term and +EGF first trimester trophoblast cultures, the fraction of IE-positive cells was measured 24 hours after a two-hour viral challenge with varying amounts of virus, expressed as MOI (taking into account the multinucleated nature of the trophoblast cultures; Figure 4.9). The results show that +EGF term and +EGF first trimester trophoblasts required >100-fold higher ratios of virus to cells for detectible infection than fibroblasts. The fraction of IE-positive fibroblasts increased to 100% with an MOI of 3.5. However, fewer than 20% of first trimester trophoblasts were infected at an MOI of 16 and only 6% of term cells at an MOI of 38. As well, the progression of infection as determined by the ratio of pp65-positive foci to IE-positive foci was considerably slower in term trophoblast cultures and intermediate in first trimester cultures compared to placental fibroblasts (Figure 4.10). Thus, not only did trophoblasts require higher virus concentrations for detection of a productive interaction to an IE-positive stage than fibroblasts, the infection in trophoblast cultures also progressed at a slower rate than in fibroblast cultures.

4.2.7 Permissive infection of trophoblasts was not unique to strain AD169

To determine if other strains of HCMV could permissively infect trophoblasts, cultured cells were challenged with AD169, two other laboratory strains (Davis and Towne; Mocarski, 1996) and a low passage clinical isolate from a congenitally infected infant. Permissive infection was evaluated using the criteria of IE and pp65 antigen expression and production of infectious virus 12 days after viral challenge. All strains

permissively infected trophoblasts, albeit to differing degrees and >99% of infectious progeny virus was cell-associated (Table 4.1). The strain variability (AD169 ~ Towne > Davis ~ congenital isolate) was reproducible in three independent experiments using trophoblasts from different placentas.

4.3 SUMMARY OF RESULTS

4.3.1 Villous trophoblasts isolated from term and first trimester placentas could be permissively infected with laboratory HCMV strain AD169.

4.3.2 Almost all infected cells in the trophoblast cultures were vimentin negative, thus trophoblasts.

4.3.3 Progeny virus remained predominantly cell-associated with little release into the trophoblast apical culture supernatant.

4.3.4 A viral challenge using an MOI of 1.0 for two hours produced an infection in +EGF and –EGF cultures that was equally inefficient when compared to infection of fibroblast cultures.

4.3.5 Trophoblast infection required a high virus to cell ratio and progressed at a slower rate compared to that of fibroblasts, with the majority of trophoblasts remaining resistant to infection.

4.3.6 Trophoblasts could be permissively infected using HCMV strains other than AD169 with varying efficiency.

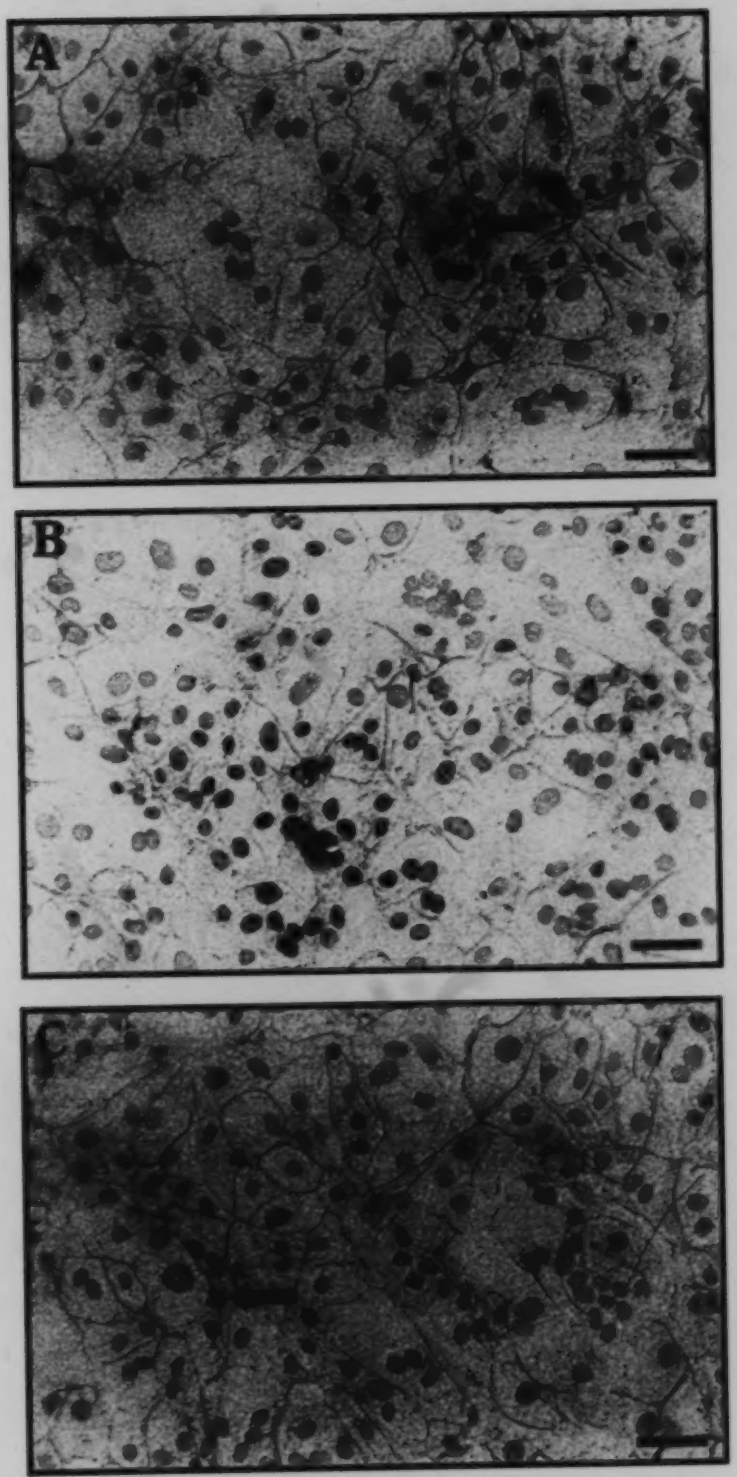

A
B
C

Figure 4.1 Detection of desmoplakin and nuclear expression of HCMV IE or pp65 antigens in trophoblast cultures by double immunohistochemical staining.
Villous trophoblasts isolated from term placentas and cultured 5 days with EGF were challenged for two hours with AD169 at an MOI of 1.0. At 12 days after viral challenge cultures were immunohistochemically stained for HCMV antigens with Ni-DAB substrate and for desmoplakin with AEC substrate. **(A)** Infected culture stained for HCMV IE antigen and desmoplakin, black arrow denotes a single IE-negative nucleus lying under an IE-positive multinucleated cell; **(B)** infected culture stained for pp65 and desmoplakin; **(C)** infected culture stained for desmoplakin, black arrow denotes a nucleus lying beneath a desmoplakin-positive cell boundary. Bar, 25 μm.

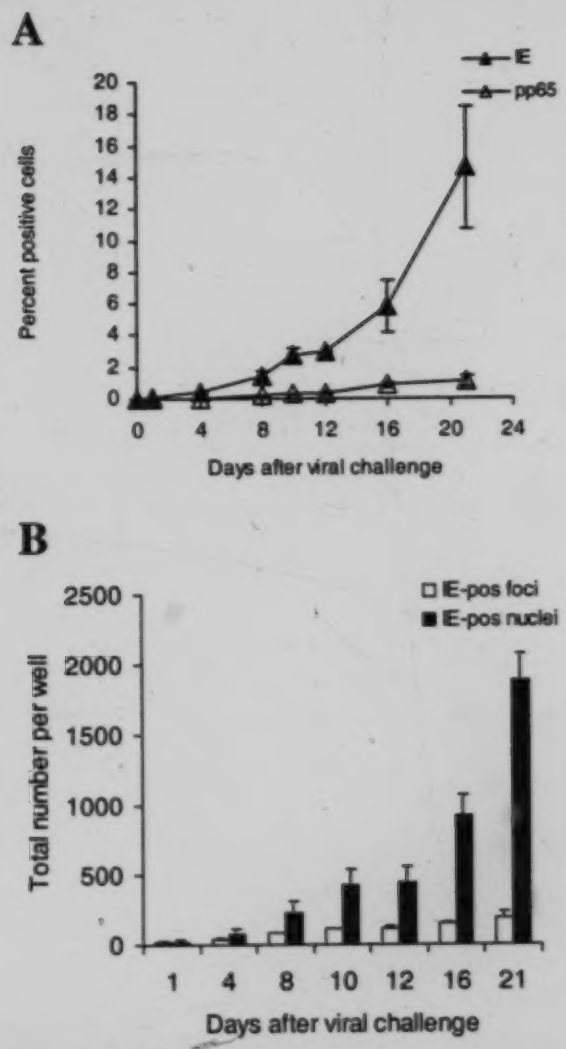


Figure 4.2 Expression of HCMV IE and pp65 antigens in term +EGF cultures as a function of time after viral challenge.

Villous trophoblasts isolated from term placentas and cultured 5 days with EGF were challenged for two hours with AD169 at an MOI of 1.0. **(A)** After further culture for the indicated periods of time (horizontal axis) cultures were stained for HCMV IE and pp65 antigens (in separate wells). The number of positive nuclei counted per well and the total number of nuclei per well estimated from DNA content were used to calculate percent positive cells by adjusting these values to reflect the multinucleated nature of +EGF cultures (based on an estimate of four nuclei per cell; see Materials and Methods specific to Chapter 4.0). Each point represents the mean ± SD of nine replicate wells and are representative of seven independent experiments. **(B)** The total number of IE-positive nuclei or IE-positive foci per well was enumerated. Each point represents the mean ± SD of four replicate wells and are representative of two independent experiments. Where error bars are not seen, error was less than the width of the marker or zero.

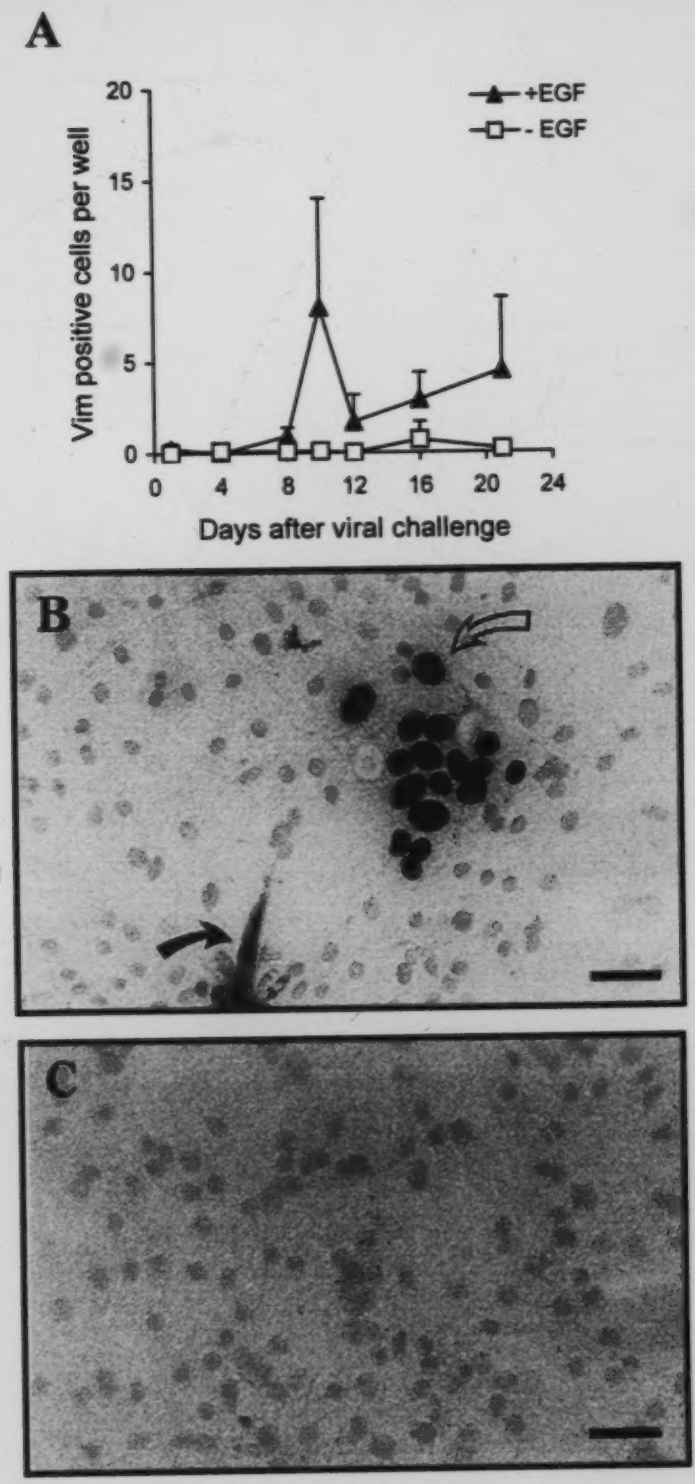

A
+EGF
- EGF
Vim positive cells per well
20
15
10
5
0
0
4
8
12
16
20
24
Days after viral challenge
B
C

Figure 4.3 HCMV infection of placental cultures was predominantly trophoblastic. Villous trophoblasts isolated from term placentas were cultured with EGF for 5 days (+EGF) or without EGF for 1 day (–EGF). **(A)** The cells were challenged for two hours with AD169 at an MOI of 1.0 and at the indicated times (horizontal axis) each well was double stained for HCMV IE antigen using Ni-DAB substrate and vimentin using AEC substrate or stained for vimentin alone. The mean ± SD of total vimentin-positive cells (Vim) in nine replicate wells was plotted against time after viral challenge in one representative experiment out of seven performed on seven different trophoblast preparations. Where error bars are not seen, error was less than the width of the marker or zero. **(B)** Infected +EGF culture at 12 days after viral challenge was stained for HCMV IE antigen (open arrow) and vimentin (closed arrow). **(C)** Infected +EGF culture at 12 days after viral challenge stained for IgG_{2a} and IgG_1, isotype controls for HCMV IE and vimentin, respectively. Bar, 25 μm.

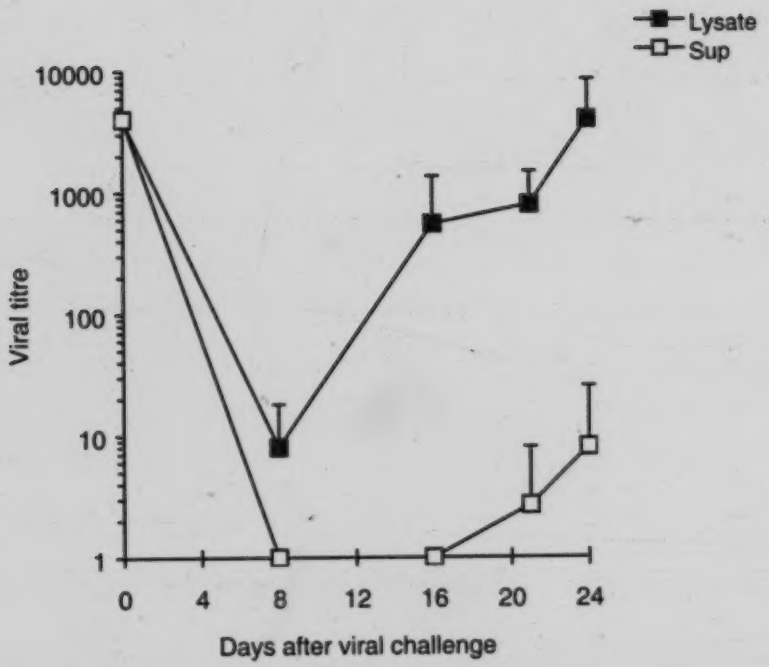


Figure 4.4 Infectious progeny virus in +EGF culture supernatants and cell lysates as a function of time after viral challenge.
Villous trophoblasts isolated from term placentas and cultured 5 days with EGF were challenged for two hours with AD169 at an MOI of 1.0. At the indicated times (horizontal axis) after challenge, 100 µL of supernatant (Sup) was removed and the adherent layer washed with PBS. The cells were lysed in 100 µL of medium by freeze-thawing (Lysate) and viral titre (PFU/mL; vertical axis) was calculated using the PFU assay on HEL fibroblasts (see Methods Specific to Chapter 4.0). Each point is the mean ± SD of three replicate wells and are representative of two independent experiments. Where error bars are not seen, error was less than the width of the marker or zero.

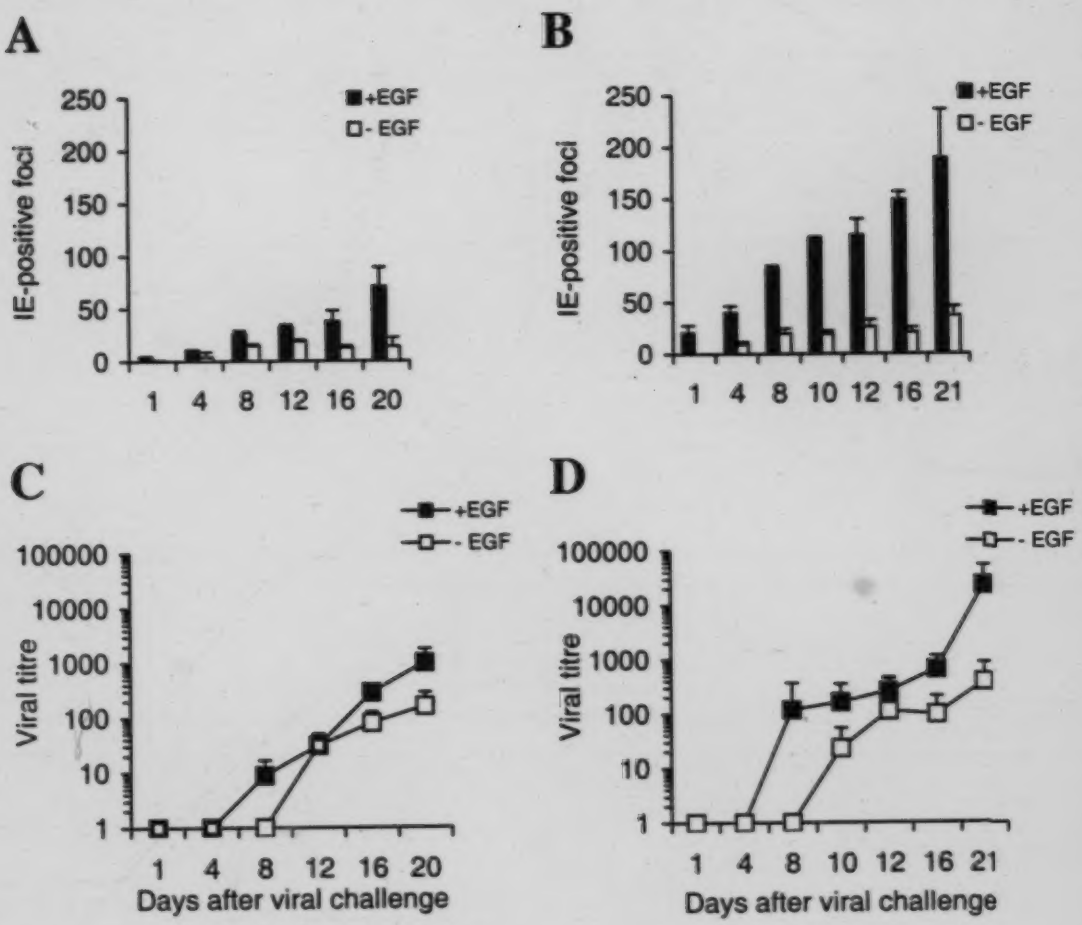


Figure 4.5 Appearance of IE-positive foci and release of progeny virus as a function of time.

Panels **A/C** and **B/D** depict a repetition of the same experiment carried out on a single prepraration of trophoblasts from a term placenta. Villous trophoblasts were cultured with EGF for 5 days (+EGF) or without EGF for 1 day (-EGF) prior to challenge for two hours with AD169 at an MOI of 1.0. Panels **A** and **B** show the number of IE-positive foci, determined by counting tight clusters of IE-positive nuclei as one foci, as a function of culture time. Panels **C** and **D** show supernatant progeny virus titers (PFU/mL), determined by assay on HEL fibroblasts, as a function of time. Each bar or point represents the mean ± SD of three replicates. Where error bars are not seen, error was less than the width of the marker or zero.

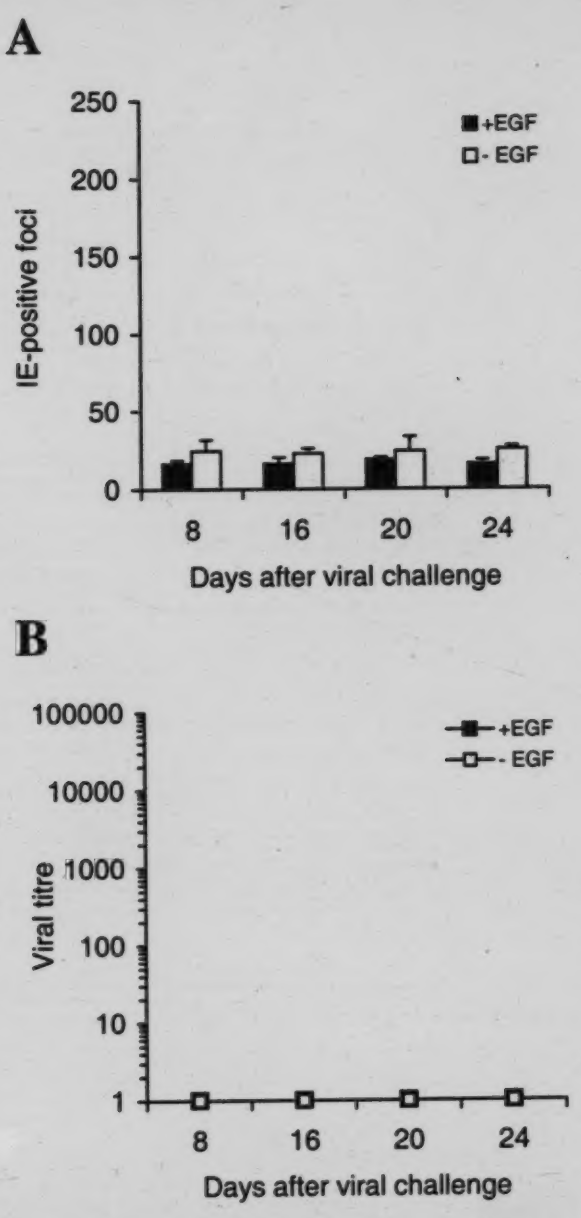


Figure 4.6 Appearance of IE-positive foci and release of progeny virus as a function of time in a second trophoblast preparation.

Villous trophoblasts from a second trophoblast preparation were cultured and challenged with HCMV as in Figure 4.5. **(A)** The number of IE-positive foci determined by counting tight clusters of IE-positive nuclei as one foci as a function of culture time. **(B)** Supernatant progeny virus titers (PFU/mL), determined by assay on HEL fibroblasts, as a function of time. Each bar or point represents the mean ± SD of three replicates. Where error bars are not seen, error was less than the width of the marker or zero.

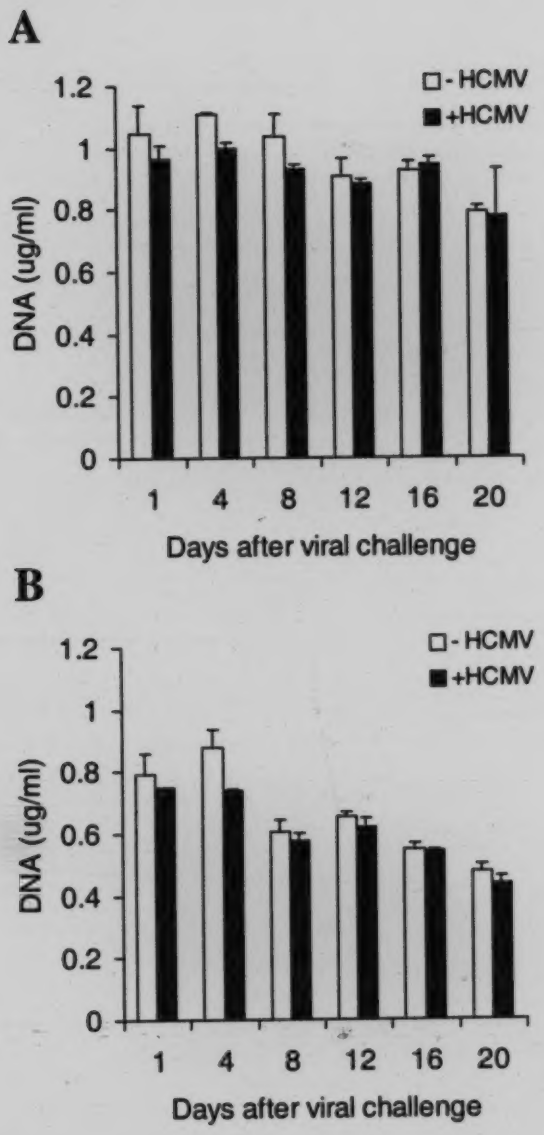


Figure 4.7 DNA content in infected and uninfected +EGF and –EGF cultures. Villous trophoblasts isolated from term placentas were cultured with EGF for 5 days **(A)** or without EGF for 1 day **(B)** and mock infected (-HCMV) or infected with AD169 (+HCMV) for two hours at an MOI of 1.0. At the indicated times (horizontal axis) after challenge, cultures were washed in PBS, lysed in ddH_2O and the DNA content determined in µg/mL using reference standards. Each bar is the mean ± SD of three replicates and are representative of two independent experiments. Where error bars are not seen, error was less than the width of the marker or zero.

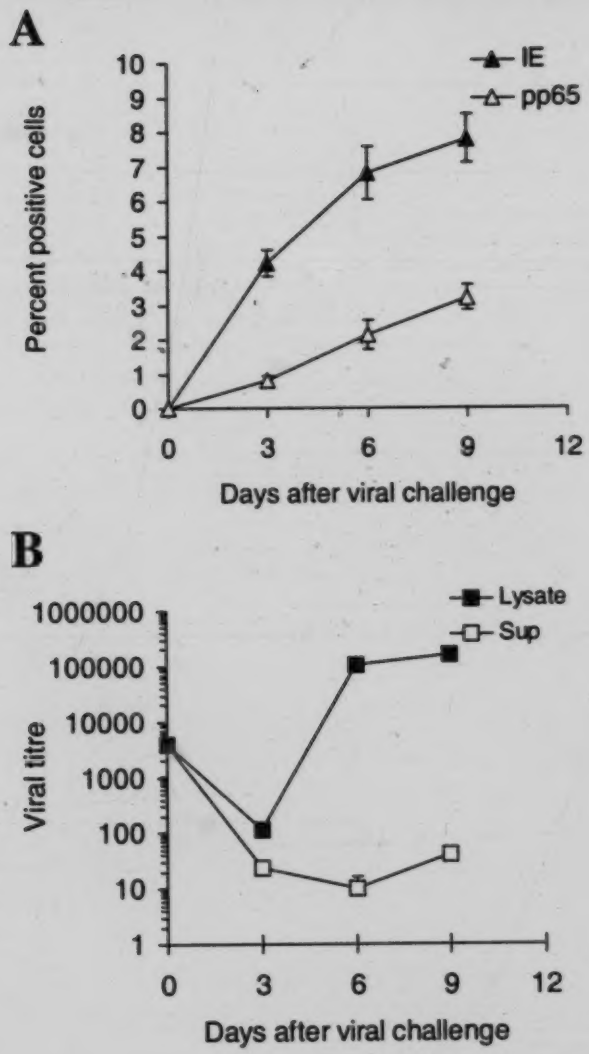


Figure 4.8 Infection of first trimester placental trophoblasts with HCMV strain AD169 as a function of time.

Villous trophoblasts isolated from first trimester placentas were cultured three days with EGF prior to challenge with HCMV for two hours at an MOI of 1.0. **(A)** At the indicated times (horizontal axis) after challenge, cultures were stained for HCMV IE and pp65 antigens and the percent infected cells was determined as described in the legend to Figure 4.2. **(B)** At the indicated times (horizontal axis), released (Sup) and cell-associated (Lysate) progeny virus titres were assessed as PFU/mL as described in the legend to Figure 4.4. The results are depicted as the mean ± SD of four replicates and are representative of two independent experiments on two different trophoblast preparations. Where error bars are not seen, error was less than the width of the marker or zero.

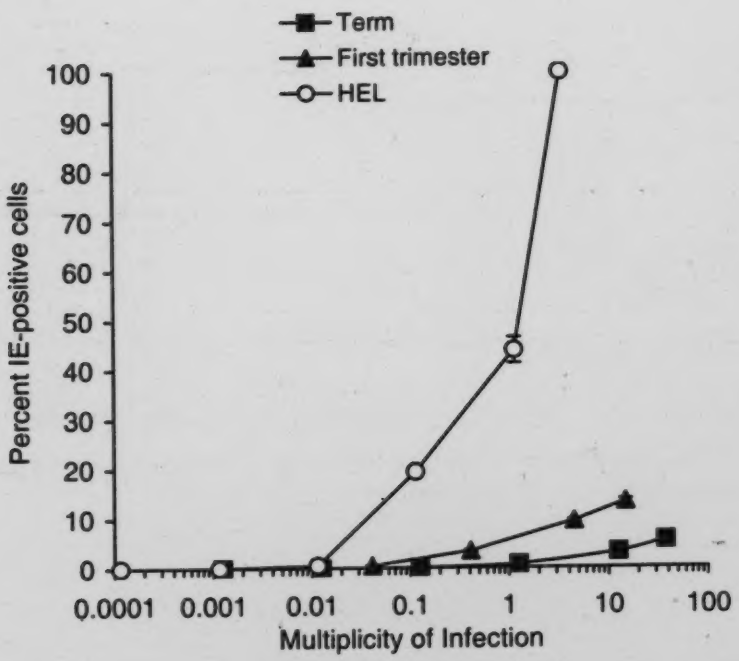


Figure 4.9 Frequency of IE-positive cells in infected cultures of HEL fibroblasts and +EGF trophoblasts from term and first trimester placentas as a function of MOI.

Villous trophoblasts isolated from term and first trimester placentas and cultured with EGF for five and three days respectively and confluent HEL fibroblasts were challenged for two hours with AD169 at the MOI indicated on the horizontal axis. The cultures were stained for IE-antigen after 24 hours of further incubation. Percent IE-positive cells in +EGF trophoblast cultures was determined as described in the legend for Figure 4.2. Percent IE-positive cells in HEL fibroblast cultures were determined by counting IE-positive cells and total cell number in five representative fields per well. Each point represents from the mean ± SD of four replicate wells and are representative of two independent experiments. Where error bars are not seen, error was less than the width of the marker or zero.

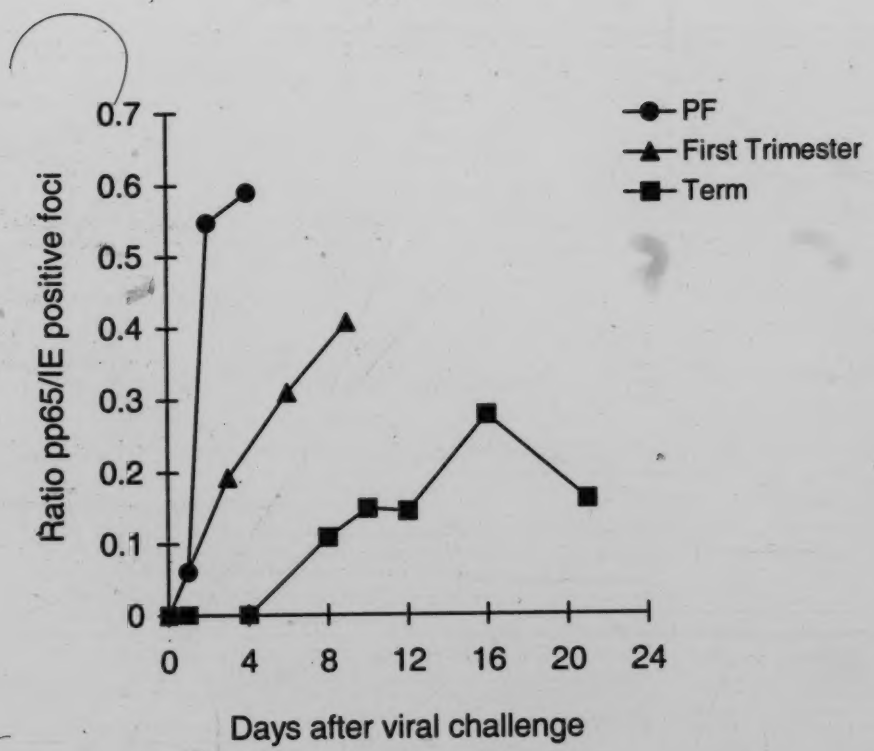


Figure 4.10 Progression of infection from IE to pp65 antigen expressing stages in placental fibroblasts and first trimester and term trophoblasts as a function of time. Villous trophoblasts isolated from term and first trimester placentas were cultured with EGF for five and three days respectively. Placental fibroblasts (PF) isolated from first trimester placentas were cultured to confluence. The trophoblasts and fibroblasts were challenged with AD169 for two hours at MOIs of 1.0 and 0.19, respectively, cultured for the indicated periods of time (horizontal axis), and stained for HCMV IE and pp65 antigens (in separate wells). IE and pp65-positive foci per well were evaluated as described in the legend to Figure 4.5 and expressed as the ratio of pp65- to IE-positive foci from the means of three replicate wells for each antigen and are representative of two independent experiments.

Table 4.1 Permissive infection of term trophoblasts with various laboratory HCMV strains and a congenital isolate

HCMV strain	No. of nuclei positive for[c]:		PFU/mL of equal vol of[d]:	
	IE	pp65	Supernatant	Lysate
AD169[a]	540 ± 130	16 ± 7.6	110 ± 56[f]	30,000 ± 8,500[f]
Towne[a]	290 ± 63	19 ± 10	710 ± 530[e]	110,000 ± 36,000[e]
Davis[a]	25 ± 19	2.8 ± 0.96	<1[e]	84 ± 120[e]
Congenital Isolate[b]	54 ± 21	2.3 ± 2.1	<1[g]	200 ± 160[g]

a +EGF cultures were challenged with the laboratory HCMV strains AD169, Towne and Davis at MOIs of 1.0 for 2 hrs and further cultured for 12 days.

b –EGF cultures were challenged with a congenital isolate at an MOI of 1.0 for 2 hrs and further cultured for 12 days.

c Mean ± SD of four wells.

d Progeny virus titres were determined by assaying supernatants and cell lysates on HEL fibroblasts and reported as PFU/mL.

e Mean ± SD of six wells.

f Mean ± SD of five wells.

g Mean ± SD of three wells.

CHAPTER 5.0 THE SUSCEPTIBILITY OF CULTURED TROPHOBLASTS TO HCMV INFECTION AS A FUNCTION OF DIFFERENTIATION

5.1 INTRODUCTION

As demonstrated in Chapter 4, trophoblasts cultured with and without EGF could be permissively infected with HCMV although relatively inefficiently using viral challenge protocols developed primarily for *in vitro* fibroblast cultures. Progeny virus remained primarily cell-associated with little release into the apical supernatant. Since trophoblasts are epithelial cells with distinct apical and basolateral membranes, progeny virus could be released preferentially in a basal (towards fetus) direction. However, to further investigate this possibility, it was essential to develop non-damaging viral challenge protocols that would result in optimal infection particularly in +EGF cultures, since these would be used to develop a testable barrier culture model resembling the villous ST (see Chapter 7.0).

Although this virus is able to attach and enter almost any cell type (Fortunato *et al.*, 2000), the first step in a permissive HCMV infection is the expression of α or immediate early (IE) proteins followed by β or delayed early and γ or late proteins and each is dependent on successful expression of the preceding group of proteins (reviewed in Mocarski, 1996). Although IE antigen can be expressed in abortive as well as permissive infections in at least some cell types (Rice *et al.*, 1984; Weinshenker *et al.*, 1988; Cinatl *et al.*, 1994; Sinzger *et al.*, 1996; Gerna *et al.*, 2000), it is useful to compare the relative frequency of IE-positive nuclei as a measure of initial infection levels. Thus the strategies undertaken to optimize the initial infection efficiency in trophoblast cultures were evaluated by measuring the frequency of IE-positive nuclei and the results of these studies are reported in the first part of this chapter.

While investigating optimization of viral challenge protocols, the number of IE-positive nuclei in infected, relatively immature –EGF cultures appeared to be greater than an equivalent infection in the more differentiated +EGF cultures. As this appeared to contradict results observed in other cell types where susceptibility to infection was dependent on a more differentiated state (Gonczol *et al.*, 1985; Smith, 1986;

I felt further investigation was warranted. As well, the overall objective of my thesis was to test the hypothesis that vertical transmission of HCMV could occur via basal release of progeny virus from infected trophoblasts of the chorionic villi. Within the intervillous space, infection from maternal blood would have to occur first in the more differentiated ST layer followed by progression of the infection through to the less differentiated CTs. Defining infection parameters and characteristics in cultures modeling each of these differentiation states was crucial to any further studies of basal release. Consequently, the last part of this chapter examines differences in susceptibility of +EGF and –EGF trophoblast cultures to HCMV infection.

5.2 RESULTS

5.2.1 HCMV infection efficiency increased with increased viral challenge time

Using mouse CMV to infect mouse embryonic fibroblasts investigators were able to demonstrate an increased number of infection plaques as a function of increased virus adsorption time (Hodgkin *et al.*, 1988). Therefore, the first approach in optimizing initial infection levels in trophoblast cultures was to increase viral adsorption or challenge time, that is, the amount of time virus is in contact with cells prior to washing. At the same time, since the lack of serum during a 24-hour viral challenge period could induce cellular apoptosis (Jung *et al.*, 1996; Valentinis *et al.*, 1998; Gerber *et al.*, 1998) and reduce the stability of HCMV at 37°C (Vonka, Benyeshmelnick, 1966), infection efficiency was compared and culture integrity monitored in +EGF cultures inoculated in serum-free medium or medium containing 2% or 10% FBS (Figure 5.1). Increasing the time of viral challenge from 2 to 24 hours generated a 3.5 to 4.5-fold increase in initial infection levels as monitored by IE-antigen expression (Figure 5.1A). The highest infection levels were observed in cultures with a viral challenge time of 24 hours in the absence of serum but there was a consistent loss of nuclei at this time point compared to cultures challenged in 2% or 10% FBS (Figure 5.1B). Viral challenge of fibroblasts in the presence of 2% FBS rather than 10% FBS has been reported to result in enhanced infection levels (Leonardi, Lipson, 1992).

At the time these studies were undertaken, a modification in stock virus isolation by using sonication rather than freeze thaw cycles to release cell-associated virus (see Materials and Methods for Chapter 5.0) resulted in increased stock viral titres of at least one log. This may also have contributed to the improved infection levels observed in this and subsequent chapters compared to that observed in Chapter 4.0.

A further increase in infection efficiency was observed by increasing the MOI from 2.2 to 10 (compare +EGF trophoblast cultures in Figures 5.1A and 5.2A). Centrifugation of CMV onto fibroblasts has long been used to enhance infection in the research setting (Osborn, Walker, 1968) and has been adapted to the clinical setting to enhance detection of cell-free virus or infected cells from patient samples (Gleaves *et al.*, 1985). This prompted me to determine if centrifugation would increase the frequency of infection of trophoblast cultures. While an increased infection level was in fact detected

in +EGF cultures (Figure 5.2A), concomitant detection of culture damage by light microscope precluded further use. Since long-term maintenance of infected trophoblast cultures was essential to investigations into the effect of infection on the barrier function of these cells, the least damaging but most effective viral challenge protocol chosen for trophoblast cultures was a 24-hour challenge period in medium containing 2% FBS at an MOI of 10.

The optimization studies were expanded to include trophoblasts not treated with EGF and HEL fibroblasts, to determine if enhancement of infection by lengthening viral challenge time was unique in this study to cells treated with EGF. The four-fold and six-fold increases in infection levels observed in –EGF trophoblast and HEL cultures respectively after 24 hours of viral challenge suggested challenge time dependent increases in infection levels are independent of cell type or culture conditions (Figure 5.2A). HCMV is heat labile and more than 70% is inactivated within two hours when cultured at 37°C in cell-free medium containing bicarbonate as previously reported (Figure 5.2B; Vonka, Benyeshmelnick, 1966). This suggested that the virus must interact with the cells in such a way as to confer protection from heat inactivation.

5.2.2 Susceptibility to HCMV infection differed between +EGF and –EGF trophoblast cultures

When viral challenge time was increased to 24 hours, the initial infection efficiency of +EGF cultures appeared to be greatly reduced compared to that of –EGF cultures (Figure 5.2A). To confirm that increased susceptibility of –EGF cultures to HCMV infection was not an isolated event dependent on the placental preparation, trophoblasts isolated from five different placentas were examined (Figure 5.3). Since each preparation varied in the number of adherent cells despite identical numbers initially plated, separate wells were prepared to specifically assess the plating efficiency of each prior to viral challenge. The total number of nuclei per well was estimated as described in Materials and Methods for Chapter 5.0. These numbers were then used to determine the total amount of virus to be added in a consistent volume to achieve an exact MOI of 10 per nucleus for each preparation and type of culture.

Two patterns of infection emerged from this representative experiment (Figure 5.3). In all five preparations –EGF cultures were significantly ($p<0.05$) more susceptible to infection by HCMV than +EGF cultures as demonstrated by higher frequencies of IE-positive nuclei. The second pattern was one of inter-preparation differences in degree of susceptibility. The data could be grossly grouped into two clusters: preparations 2 and 5 appeared to be less susceptible in both +EGF and –EGF cultures than Preparations 1, 3 and 4.

Infection levels are reported in this and subsequent chapters as percent positive nuclei. However, comparison of infection levels in +EGF and –EGF cultures using percent positive nuclei will not be precisely valid since 35 to 60% of nuclei in +EGF cultures are multinucleated (Table 5.1; (Kilani *et al.*, 1997)). I therefore compared, in a separate experiment, the values for percent positive nuclei versus percent positive cells using a double immunohistochemical staining method for IE antigen and desmoplakin, a protein found in cell boundaries and used as a marker for syncytialization (Douglas, King, 1990). Percent positive nuclei were assessed as previously described. Percent positive cells were assessed by counting an area clearly demarcated by desmoplakin as one cell regardless of the number of enclosed nuclei. In –EGF cultures from five preparations, the percent positive nuclei value was decreased on average by 2.0 ± 0.9% when assessed for percent positive cells reflecting some multinucleation in –EGF cultures. In +EGF cultures from two preparations, the percent positive nuclei value was decreased on average by 9.8 ± 3.9% when assessed for percent positive cells reflecting the increased numbers of multinucleated cells in these cultures. These values should be kept in mind throughout the rest of the thesis. These results confirm the susceptibility differences found between infected –EGF and +EGF trophoblast cultures and suggest they may be even more pronounced when assessed on the basis of percent positive cells rather than nuclei.

5.2.3 HCMV infection levels in +EGF cultures were not affected by the presence of EGF during viral challenge

As viral challenge of +EGF cultures was done in medium containing EGF, it was essential to determine if the presence of EGF during challenge affected initial infection

levels. +EGF and –EGF trophoblast and HEL fibroblast cultures were challenged with HCMV for 24 hours in 2% FBS medium with or without EGF and the frequency of IE-positive nuclei was monitored (Figure 5.4). In fibroblast and –EGF trophoblast cultures initial infection levels increased 1.5- and 3.2-fold respectively if EGF was present during challenge. In +EGF cultures, however, no difference in infection levels was noted whether EGF was present during challenge or not. Thus, as a precaution to ensure maintenance of healthy cultures, trophoblast cultures pretreated with EGF were always challenged in the presence of EGF.

5.2.4 Trophoblast susceptibility to HCMV infection decreased with increased time in culture

Trophoblasts cultured for five days in the presence of EGF resemble the more differentiated multinucleated ST whereas trophoblasts cultured without EGF for one day resemble the immature mononucleated CT (Yui *et al.*, 1994; Morrish *et al.*, 1997). Both cultures continue to mature over time as determined by increasing expression of differentiation markers and although –EGF cultures remain predominantly mononucleated, +EGF cultures develop increasingly extensive syncytium (Morrish *et al.*, 1987; Yui *et al.*, 1994; Morrish *et al.*, 1997). If a relatively immature state of differentiation at the point of viral challenge is important for susceptibility to HCMV infection as reflected by the results of the previous section, trophoblasts that have aged and further matured in culture should be less susceptible to infection regardless of culture treatment. Trophoblasts cultured with or without EGF for various lengths of time prior to challenge with identical amounts of virus were examined for expression of IE antigen (Figure 5.5A). As predicted, HCMV susceptibility decreased as a function of trophoblast time in culture prior to viral challenge with a more pronounced effect observed in the +EGF cultures. Decreased susceptibility was not as a result of changes in the number of nuclei in each infected culture (Figure 5.5B).

Since expression of IE antigen and thus susceptibility to HCMV infection could also be dependent on the physiological state of the host cell (DeMarchi, Kaplan, 1977), metabolic capacity was monitored by MTT assay over time in uninfected +EGF or –EGF cultures. Since metabolic activity in both cultures was maintained over long periods of

time with consistently higher values in the +EGF cultures (Figure 5.6), it did not appear to explain differences in HCMV susceptibility.

5.2.5 Susceptibility to HCMV infection decreased as PLAP expression increased in trophoblast cultures

From the preceding results, it appeared that the more mature a trophoblast culture, the more resistant to HCMV infection. To more clearly define any relationship between differentiation and HCMV susceptibility, the need arose for a marker that would delineate differentiated from immature trophoblasts. Placental alkaline phosphatase (PLAP) is an enzyme expressed on the apical microvillae of the highly differentiated ST and is often used to assess purity of microvillous versus basal membrane vesicles (Vanderpuye, Smith, 1987; Illsley *et al.*, 1990; Eaton, Oakey, 1994). It is not expressed on immature CT and I thus sought to investigate its usefulness as a differentiation marker in trophoblast +EGF and –EGF cultures. To determine if PLAP expression at the point of HCMV infection was associated with infection susceptibility, each of the trophoblast preparations depicted in Figure 5.3 at time points just prior to viral challenge (Day 5 for +EGF and Day 1 for –EGF) were immunohistochemically stained for PLAP or an isotype control. As seen in Figure 5.7, PLAP expression was highly variable both within a single well and between preparations with differential amounts of strong positive, intermediate positive and weak to negative areas visible. There were generally more and larger PLAP-positive areas in +EGF than –EGF cultures (compare Figure 5.7A,C with 5.7E,G). To determine if there was an association between the number of cells expressing PLAP at the time of viral challenge and initial infection levels in +EGF and –EGF cultures, trophoblast cultures from the five different placentas in Figure 5.3 were compared. A significant negative linear correlation ($p = 0.034$) was found in the +EGF cultures suggesting that a high percentage of PLAP-positive cells in a culture predicts a low infection frequency (Figure 5.8B). Although a comparable analysis for –EGF cultures was not significant ($p = 0.180$), a similar trend is evident (Figure 5.8A). In both cases the power of the analysis was substantially below (<0.3 to <0.6) the optimum of 0.8 and more placentas would have to be analyzed for any firm conclusions to be drawn.

5.2.6 Susceptibility to HCMV infection was related to the maturation state of the individual trophoblast

Although investigating HCMV infections in entire trophoblast cultures yielded useful information, it was essential to relate susceptibility to the maturation state of individual trophoblasts, particularly in view of the heterogeneous nature of these cultures. Infected –EGF cultures three days after viral challenge were examined by double immunohistochemistry for the location of IE-positive nuclei in relation to the staining intensity of PLAP-positive area in which they were found. Preliminary experiments showed that the majority of IE-positive nuclei were detected in PLAP-positive areas of intermediate to negative staining intensity (Figure 5.9A,B). Some of the very few IE-positive nuclei detected in strongly positive PLAP areas appeared to be in fact in intermediate or negative areas lying underneath strongly positive areas (see inset; Figure 5.9B). Evidence for the multilayered nature of +EGF cultures can also be found in Figure 4.1 and is evaluated more thoroughly by electron microscopy (EM) in Chapter 7.0. These results suggested that strongly positive PLAP areas were relatively resistant to HCMV infection and cultures with a high concentration of these areas (compare +EGF to –EGF cultures in Figure 5.7) would have lower infection frequencies (Figure 5.3).

To further define the state of trophoblast maturation important to HCMV susceptibility, the relationship between infection and syncytialization was examined. Infected +EGF and –EGF cultures were double stained for IE antigen and desmoplakin. The fraction of mononucleated or multinucleated cells that were IE-positive were evaluated and compared to the distribution of total cells appearing in mononucleated versus multinucleated cellular units (Table 5.1). In –EGF cultures the fraction of multinucleated cells that were IE-positive (e.g. 51.2%; 10 IE-positives out of a total of 20 multinucleated cells) was high even though only 10.6% of total cells were multinucleated. This was not the situation in +EGF cultures where even though the fraction of total cells that are multinucleated increases approximately three fold, the number of infected multinucleated cells is approximately half that found in –EGF cultures (e.g. 22.5%; 10 IE-positives out of a total of 44 multinucleated cells).

5.3 SUMMARY OF RESULTS

5.3.1 Increasing the time HCMV was in contact with cells increased initial infection levels as detected by IE-antigen expression in HEL fibroblast, +EGF and –EGF trophoblast cultures.

5.3.2 The presence of EGF during viral challenge either had no effect (+EGF cultures) or resulted in increased infection levels (-EGF trophoblast and HEL fibroblast cultures) compared to viral challenge in the absence of EGF.

5.3.3 The optimal viral challenge protocol for trophoblast cultures which resulted in high infection levels and initially healthy cultures was a 24-hour challenge time in culture medium containing 2% FBS with (+EGF cultures) or without (-EGF cultures) EGF at an MOI of 10.

5.3.4 -EGF trophoblast cultures were more susceptible to HCMV infection than +EGF cultures.

5.3.5 HCMV susceptibility decreased with increasing age of trophoblast culture with a more pronounced effect in +EGF cultures.

5.3.6 Regression analysis suggested that HCMV susceptibility negatively correlated with PLAP-positivity on a per-culture basis and preliminary work showed that strongly PLAP-positive areas may be resistant to infection.

5.3.7 Multinucleated syncytium appeared to be more susceptible to HCMV infection than mononucleated cells in –EGF cultures but not +EGF cultures.

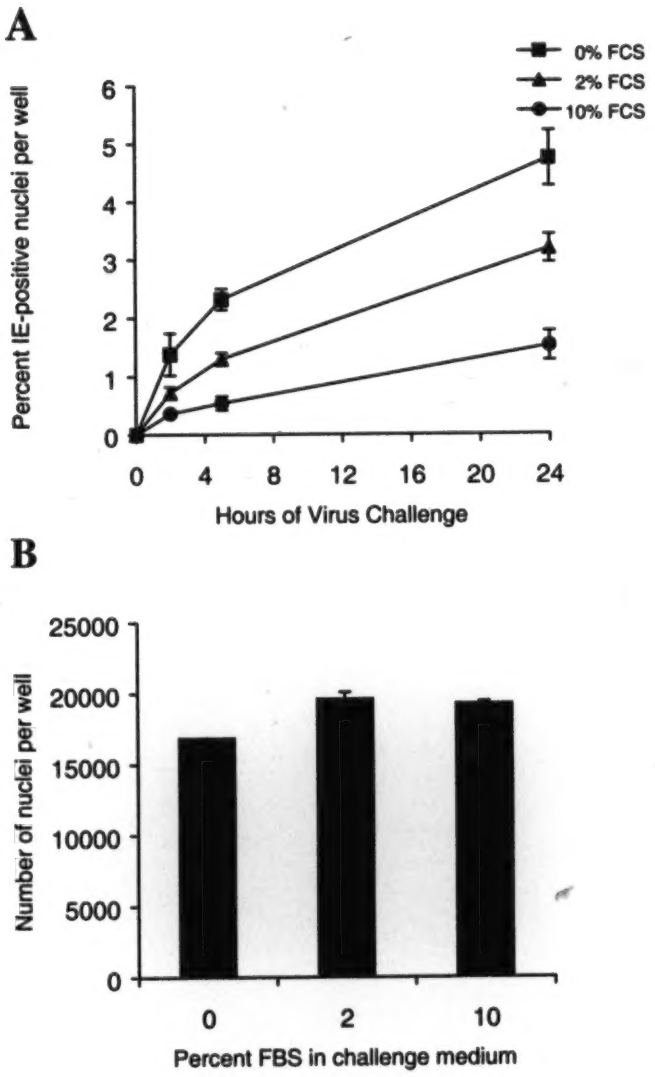


Figure 5.1 Effects of serum and increasing viral challenge times on HCMV infection efficiency in +EGF cultures.

(A) +EGF cultures were challenged with AD169 at an MOI of 2.2 for 2, 5 or 24 hrs in IMDM containing 0%, 2% or 10% FBS. After the indicated challenge time (horizontal axis), each culture was washed five times in IMDM, incubated for a further 48 hrs in 2% FBS/IMDM, stained for HCMV IE antigen and counterstained with hematoxylin. Percent IE-positive nuclei were calculated from enumeration of the total IE-positive nuclei per well divided by the total nuclei per well as estimated in (B). The mean ± SD of triplicate wells from one of two independent experiments is reported. **(B)** Total number of nuclei per well in the cultures described in (A) was determined by multiplying the average of five fields by the total number of fields per well and reported as the mean ± SD of triplicate wells. Where error bars are not seen, error was less than the width of the marker or zero.

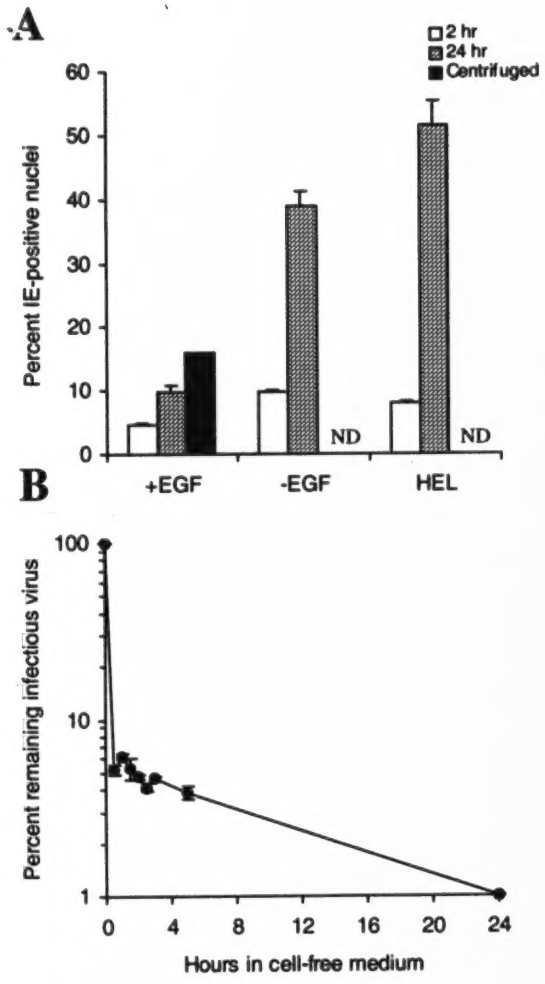


Figure 5.2 HCMV infection efficiencies in trophoblast and fibroblast cultures and viral infectivity in cell-free medium.

(A) +EGF and –EGF cultures were challenged with AD169 at an MOI of 10 for 2 or 24 hrs in 2% FBS/IMDM. After the 2 or 24-hr challenge time, each culture was washed five times in IMDM, incubated for a further 48 hours and immunohistochemically stained for IE antigen. +EGF cultures were also challenged at an MOI of 10 by centrifugation (see the Materials and Methods for Chapter 4.0). HEL fibroblasts were cultured to confluence, challenged with AD169 at an MOI of 0.2 for 2 or 24 hrs in 2% FBS/MEM, washed and incubated for a further 18 hrs. Percentages were calculated as described in the legend to Figure 5.1 and reported as the mean ± SD of triplicate wells from one of two independent experiments. ND = not done. **(B)** 2000 PFU of infectious AD169 were added to wells containing cell-free 2% FBS/IMDM. Aliquots were removed from separate wells in triplicate at each time point and tested on fresh confluent HEL fibroblasts for the presence of infectious virus. The percent of virus remaining infectious was based on the amount originally added and calculated as the mean ± SD of triplicate wells from one of two independent experiments. Where error bars are not seen, error was less than the width of the marker or zero.

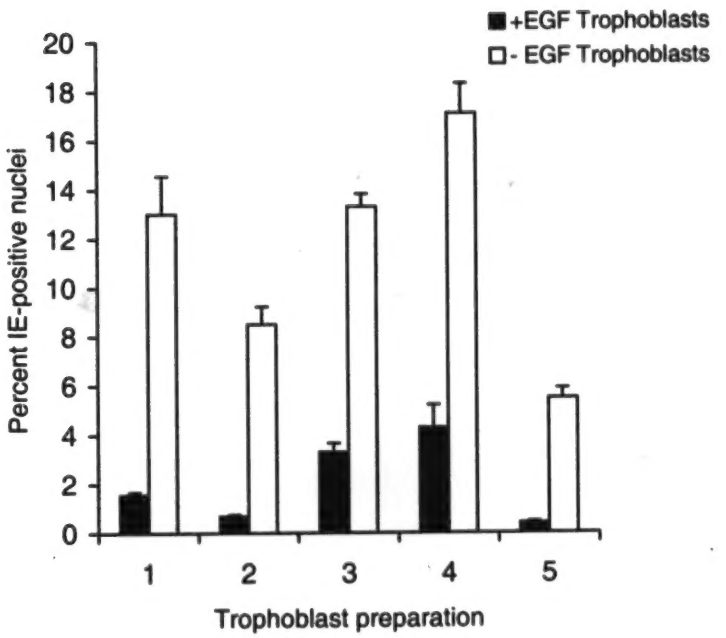


Figure 5.3 Susceptibility of +EGF and –EGF trophoblast cultures from five placental preparations to HCMV infection.
Trophoblasts isolated from five different placentas were cultured with EGF for five days (+EGF cultures) or without EGF for one day (-EGF cultures) and challenged with AD169 at an MOI of 10 per nucleus for 24 hours. After washing and a further 48-hour incubation, cultures were stained for IE antigen. Percentages were calculated as the mean ± SD of triplicate wells from one of two independent experiments. A statistically significant difference ($p<0.05$) between infected +EGF and –EGF cultures was demonstrated using the non-parametric Wilcoxon signed ranks test.

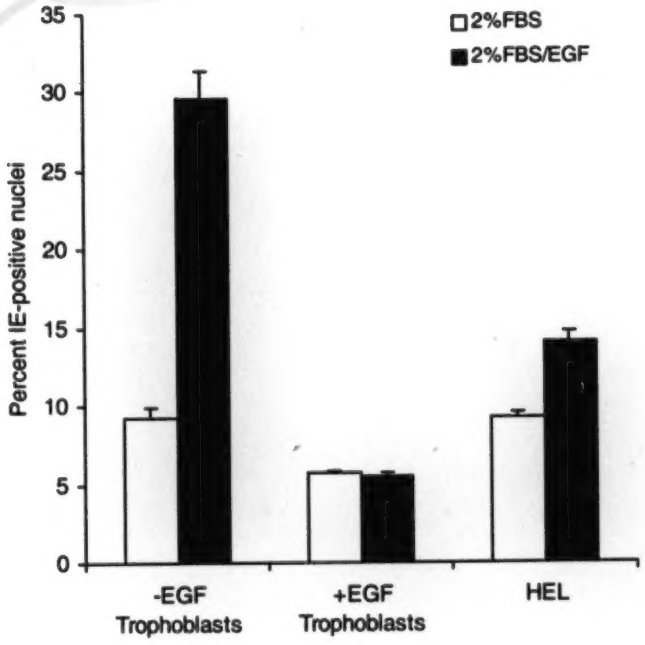


Figure 5.4 Effect of EGF during viral challenge on susceptibility to HCMV infection.
Trophoblasts were cultured with EGF for 5 days (+EGF cultures) or without EGF (-EGF cultures) for 1 day. Following extensive washing the cultures were challenged with AD169 at an MOI of 10 in the presence or absence of 10 ng/mL of EGF for 24 hrs. Viral inoculum was removed, the cultures washed and further incubated in fresh prechallenge medium for 48 hours. Confluent HEL cells were challenged with AD169 at an MOI of 0.2 in the presence or absence of 10 ng/mL of EGF for 2 hrs, washed and further incubated without EGF for 24 hrs. Cultures were stained for IE antigen and percentages calculated as the mean ± SD of triplicate wells from one of two independent experiments. Where error bars are not seen, error was less than the width of the marker or zero.

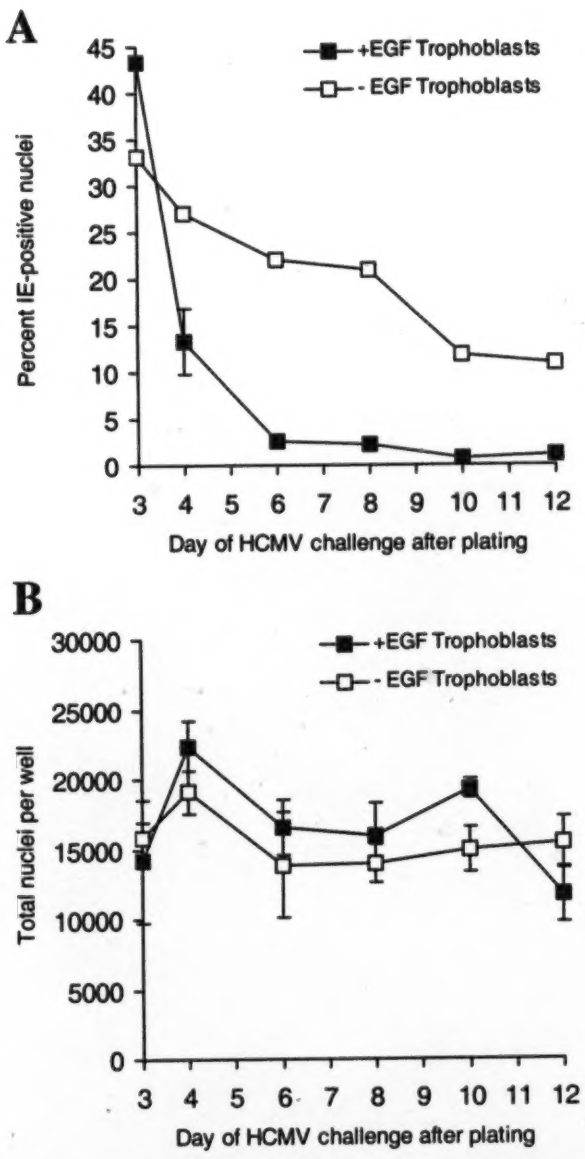


Figure 5.5 HCMV susceptibility in aging +EGF and -EGF cultures.
(A) Trophoblasts cultured with or without EGF were challenged with AD169 at an MOI of 10 at various times after plating. After a 24-hour challenge period, the cultures at each time point were washed, incubated a further 48 hours and immunohistochemically stained for HCMV IE antigen and counterstained with hematoxylin. Percentages were calculated as the mean ± SD of triplicate wells from one of two independent experiments. **(B)** The total number of nuclei per well in the cultures from (A) was calculated as for Figure 5.1. Where error bars are not seen, error was less than the width of the marker or zero.

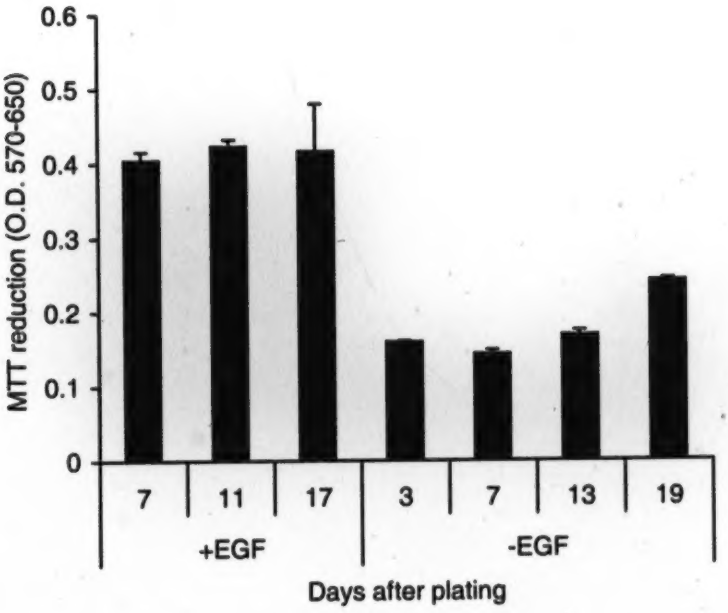
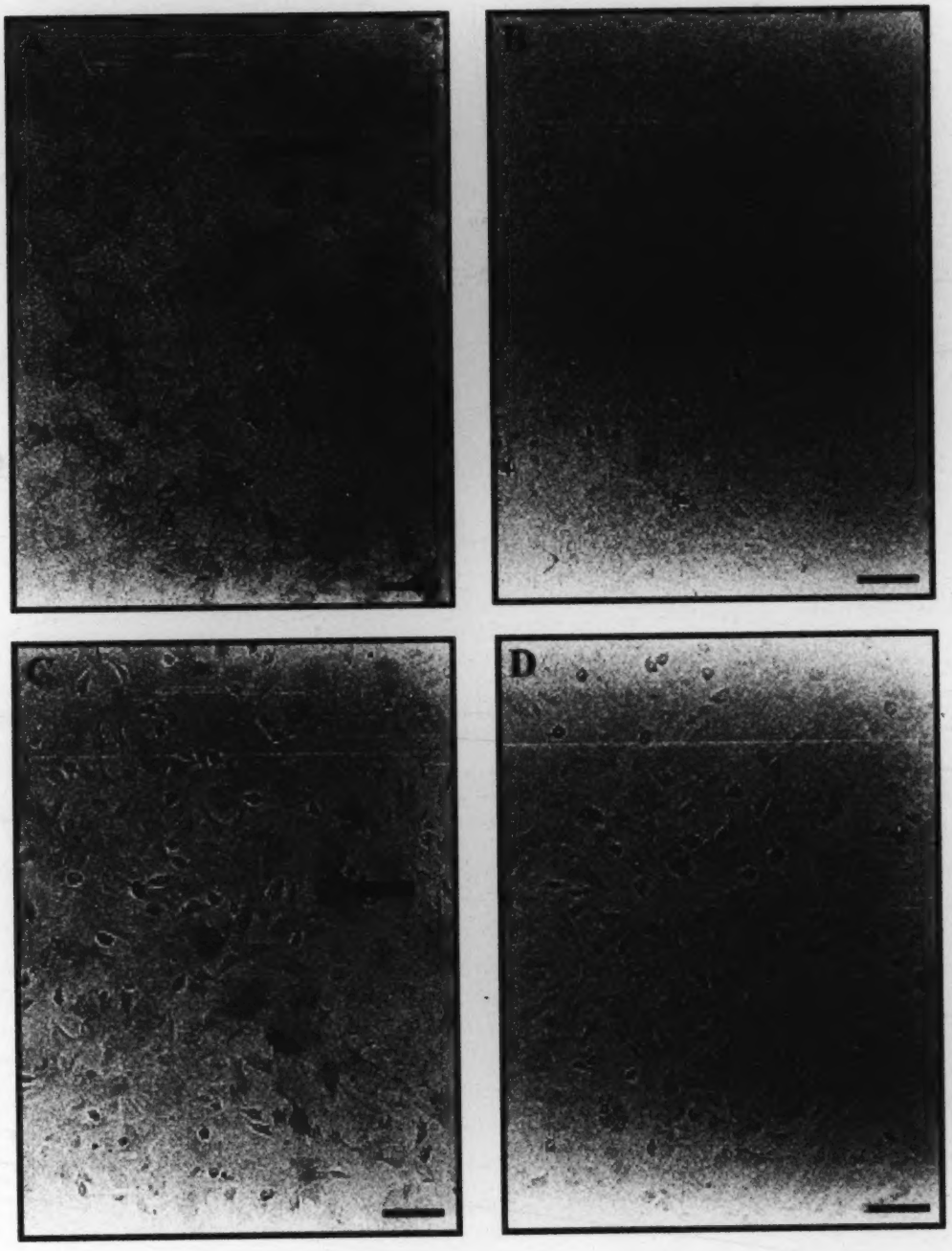


Figure 5.6 Metabolic capacity in uninfected +EGF and –EGF trophoblast cultures as a function of time.

Trophoblasts were cultured with (+EGF) or without EGF (-EGF) and at the indicated days after plating assessed for metabolic capacity by MTT assay. Each bar represents the mean in triplicate wells ± SD from one of four independent experiments. The optical density (O.D.) was adjusted for variation in the number of nuclei at each time point by reporting the amount per 20,000 nuclei per well. Where error bars are not seen, error was less than the width of the marker or zero.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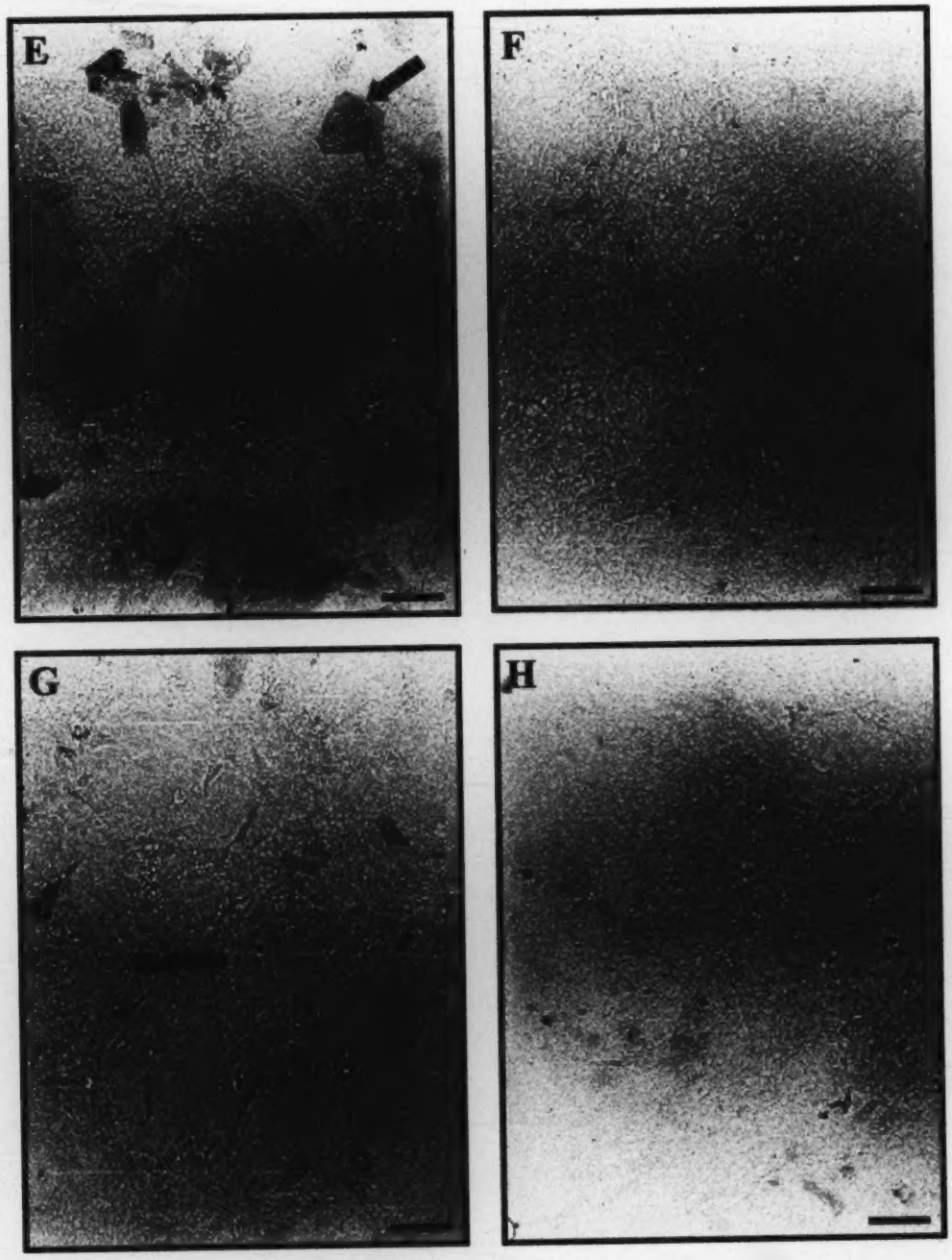
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H

Figure 5.7 PLAP expression in placental preparations with high and low susceptibility to HCMV infection.
Trophoblasts from Preps 5 (A,B,E,F) and 3 (C,D,G,H) were cultured with EGF for five days (E,F,G,H) or without EGF for one day (A,B,C,D) and immunohistochemically stained for PLAP (A,C,E,G) or an isotype control, IgG_{2a} (B,D,F,H). The solid black arrows depict strong PLAP-positive areas. Bar, 25 μm.

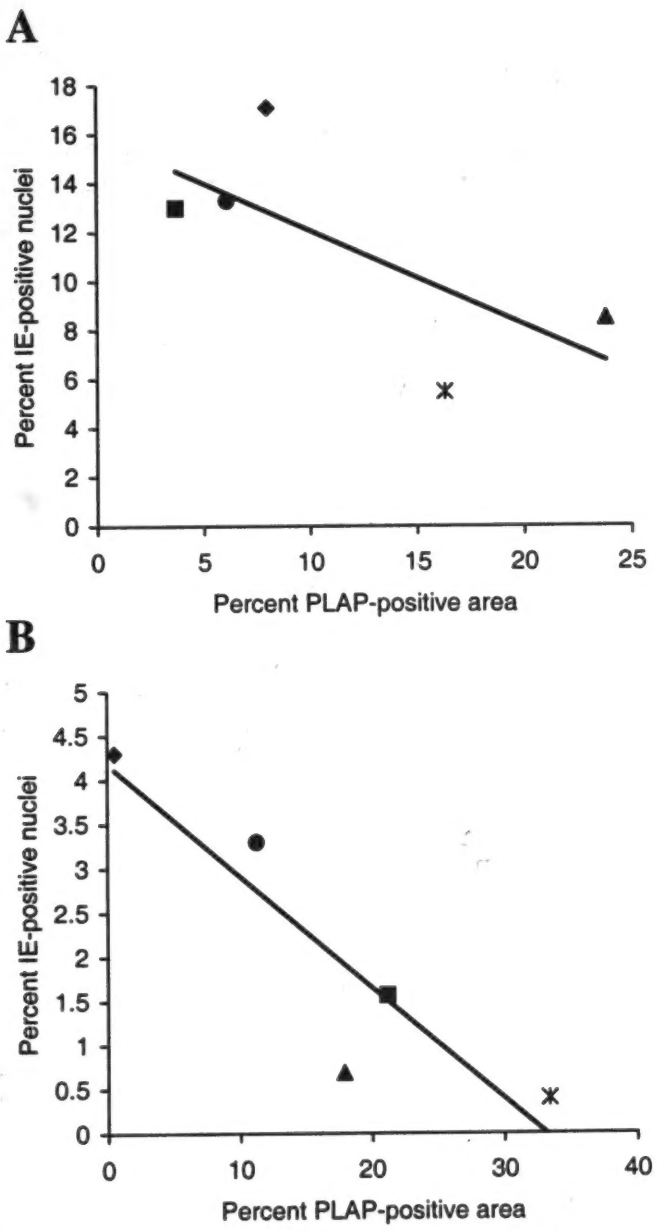


Figure 5.8 Correlation of infection levels to PLAP-positive levels in +EGF and –EGF cultures from five placental preparations.
The association of infection levels depicted in Figure 5.3 and the percent-PLAP positive levels just prior to viral challenge were assessed by linear regression analysis in **(A)** –EGF cultures; R = 0.709, p = 0.180 and **(B)** +EGF cultures; R = 0.906, p = 0.034 where R is the regression coefficient. Each symbol represents the placental preparation as depicted in Figure 5.3: 1 = square, 2 = triangle, 3 = circle, 4 = diamond and 5 = star.

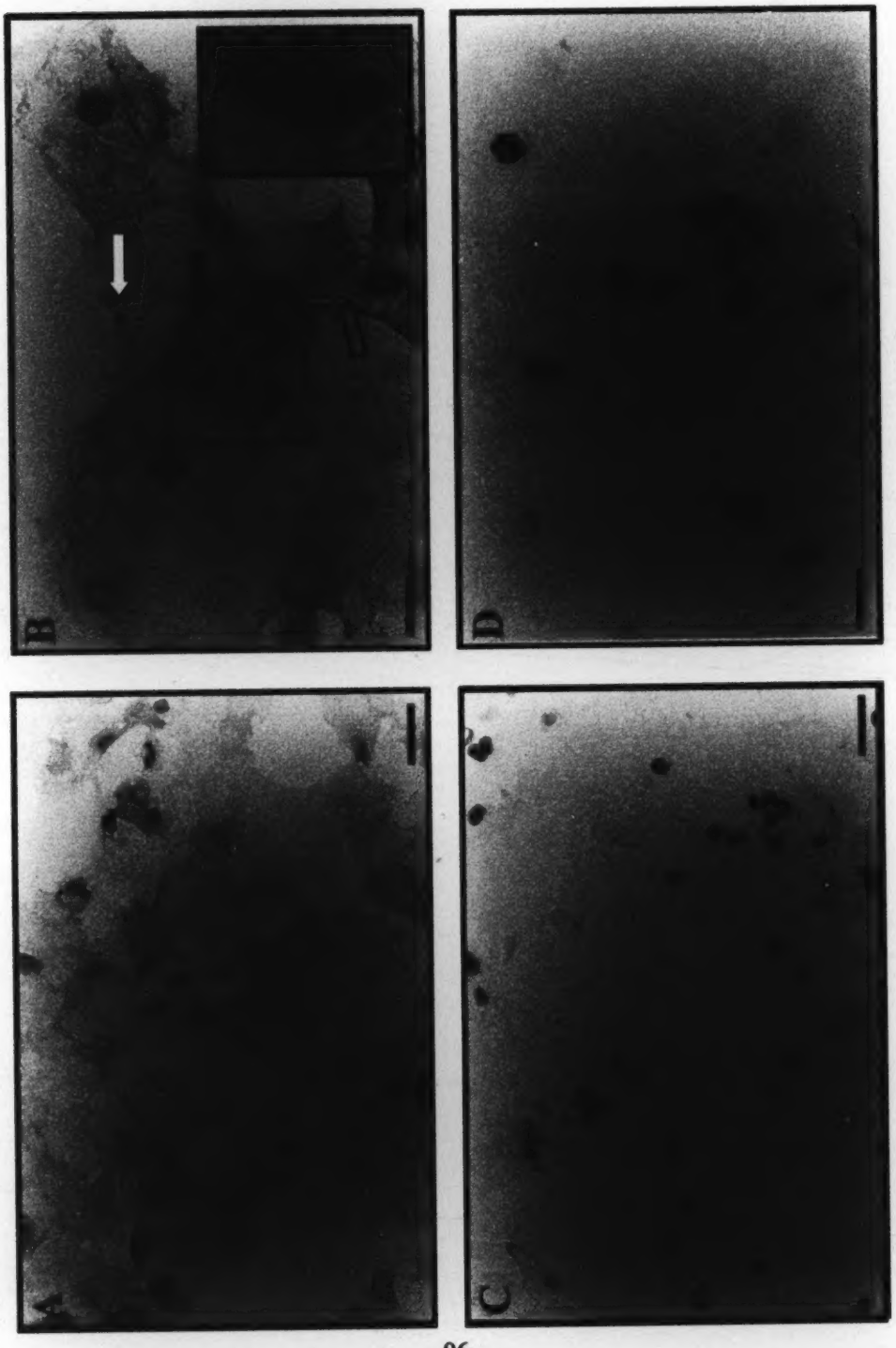

B
D
A
C

Figure 5.9 Colocalization of IE-positive nuclei in intermediate to negative PLAP-positive areas.

-EGF trophoblasts cultured and challenged as in Figure 5.3 were double stained for IE-antigen and PLAP **(A,B)** or IE antigen and IgG_{2a} control antibody **(C,D)** using Ni-DAB and AEC respectively. Arrows depict the following: solid black arrow = strongly PLAP-positive area, open arrow = intermediate PLAP-positive area, white arrow = PLAP-negative area. Inset picture depicts an IE-positive nucleus lying underneath a strongly PLAP-positive area. Bar = 25 μm (A,C) and 10 μm (B,D).

Table 5.1[a] Relationship of HCMV susceptibility to multinucleated state of trophoblast cultures.

	(A) % mononucleated cells[c]	(C) % IE-positive mononucleated cells[d]	(B) % multinucleated cells[c]	(D) % IE-positive multinucleated cells[d]
-EGF[b]	89.4 ± 3.1	14.2 ± 1.2	10.6 ± 3.1	51.2 ± 5.4
+EGF	65.2 ± 1.0	18.4 ± 2.9	34.7 ± 1.0	22.5 ± 1.3

a Results are representative of three independent experiments.

b +EGF and –EGF cultures were challenged with AD169 at an MOI of 10 for 24 hrs and cultured for an additional 48 hrs. Dual immunohistochemical staining for IE antigen and desmoplakin were performed.

c The fraction of mononucleated (A) or multinucleated (B) cells to total cells was evaluated per well and the mean percent ± SD determined for three wells.

d The fraction of mononucleated (C) or multinucleated (D) cells that were IE-positive were evaluated and the mean percent ± SD determined for three wells.

CHAPTER 6.0 HCMV INFECTION PROGRESSION IN +EGF AND –EGF TROPHOBLAST CULTURES

6.1 INTRODUCTION

Although HCMV can enter many different cell types (Fortunato *et al.*, 2000), the outcome of infection is dependent on host cell factors and virus strain (Rice *et al.*, 1984; Gonczol *et al.*, 1985; Turtinen *et al.*, 1987). Possible outcomes include: (a) fully permissive infections with production of progeny virus and cytopathology, (b) abortive infections where IE antigens may or may not be expressed but no progeny virus is produced (Rice *et al.*, 1984; Weinshenker *et al.*, 1988; Cinatl *et al.*, 1994; Sinzger *et al.*, 1996; Gerna *et al.*, 2000) or (c) latent infections where the virus enters a state of quiescence essentially hidden within the cell, the mechanistic details of which are still largely unknown (Taylor-Wiedeman *et al.*, 1991; Minton *et al.*, 1994; Fish *et al.*, 1998; Hahn *et al.*, 1998).

The state of maturity is one of the host cell factors important to development of permissive HCMV infections and with a few exceptions, including infection in fibroblasts (Smith, 1986; Poland *et al.*, 1990), advanced differentiation has been shown to be important both *in vivo* (Pulliam, 1991; Sinzger *et al.*, 1996) and *in vitro* (Tanaka *et al.*, 1984; Gonczol *et al.*, 1985; Smith, 1986; Weinshenker *et al.*, 1988; Poland *et al.*, 1990; Lathey, Spector, 1991; Ibanez *et al.*, 1991; DiLoreto *et al.*, 1994; Tugizov *et al.*, 1996). Fully permissive infections can be induced in primary cells or cell lines upon treatment with agents that stimulate differentiation such as hydrocortisone (Ibanez *et al.*, 1991), cocultivation with activated T cells (Lathey, Spector, 1991), phorbol esters (Weinshenker *et al.*, 1988) and DMSO (Tanaka *et al.*, 1985). In a neuronal cell line, although undifferentiated cells can be permissively infected, differentiation results in more extensive infection with a higher yield of progeny virus (Poland *et al.*, 1994). Colon-derived intestinal epithelial cells (Caco-2) and rhabdomyosarcoma cells represent two exceptions: poorly differentiated cells can be permissively infected while highly differentiated cells either express only IE antigens (Cinatl *et al.*, 1994; Jarvis *et al.*, 1999) or can also be permissively infected (Esclatine *et al.*, 2000). Compared to fibroblasts, other primary human cells and cell lines that are permissive for HCMV generate an

inefficient infection (Michelson-Fiske *et al.*, 1975; Knowles, 1976; Tumilowicz *et al.*, 1985; Smith, 1986; Lathey *et al.*, 1990; Poland *et al.*, 1990).

Egress of this virus from a permissively infected fibroblast likely follows an exocytic pathway through the Golgi apparatus since it is sensitive to brefeldin A (Eggers *et al.*, 1992). Progeny virus is usually efficiently released from infected cells so that late in infection half of the progeny virus is found in the supernatant with half remaining cell-associated. In many cell types progression of the infection to cell lysis is evident (Poland *et al.*, 1990; Kahl *et al.*, 2000) or occurs but is delayed (Tumilowicz *et al.*, 1985). In other cells, lysis does not occur: e.g. in macrophages progeny virus is preferentially retained in cytoplasmic vesicles (Fish *et al.*, 1995; Fish *et al.*, 1998). Loss of the infected cell is unlikely to be due to programmed cell death since HCMV infection appears to block apoptosis (Zhu *et al.*, 1995; Cinatl *et al.*, 1998; Lukac, Alwine, 1999).

In the previous chapter, susceptibility to the IE stage of HCMV infection was shown to be dependent on the differentiation state of the cell with greater infection in the more immature –EGF cultures than the more differentiated +EGF cultures. As stated previously IE antigen expression may reflect either an abortive or permissive infection (Rice *et al.*, 1984; Weinshenker *et al.*, 1988; Cinatl *et al.*, 1994; Sinzger *et al.*, 1996; Gerna *et al.*, 2000). Thus it was important to follow the progression of infections generated using the optimal protocols developed in the previous chapter to verify permissiveness and confirm the original observation of predominantly cell-associated progeny virus. These results are described in this chapter.

Although infection in differentiated +EGF cultures was permissive, infection in immature trophoblast (-EGF) cultures progressed more rapidly with increasing numbers of cells positive for IE and pp65 proteins and greater production of progeny virus. The differences were found to be independent of EGF itself but dependent on the maturation state at the time of initial infection.

6.2 RESULTS

6.2.1 Low pH or IVIG treatment after viral challenge removed adherent virus

During the optimization studies in the previous chapter, I discovered that although cultures were vigorously washed after the 24-hour challenge time and little or no infectious virus was detected in the final wash (i.e. 303 ± 125 PFU per well in +EGF cultures and 312 ± 83 PFU per well in –EGF cultures), significant infectious virus was detected in culture supernatants 24 to 48 hours later (Day 2, IMDM wash; Table 6.1). This amounted to 1.36 ± 0.28 % of the original inoculum in +EGF cultures and 0.54 ± 0.08 % in –EGF cultures. The same phenomenon was not observed after two-hour challenge times at lower MOIs (Day 1 and 4; Figure 4.5C and 4.5D) or after 24-hour challenge of fibroblasts (Table 6.2). Since virus production in trophoblasts is delayed until at least the fourth day after challenge (Figure 4.4), infectious virus found one or two days after viral challenge may be due to initially adherent residual inoculum that is eventually released into the supernatant. Reversible binding of murine CMV to mouse embryo fibroblasts has previously been demonstrated (Hodgkin *et al.*, 1988).

Removal of residual inoculum directly after initial viral challenge and removal of potentially adherent virus prior to assessment of cell-associated virus at various times after viral challenge was essential to accurately interpret viral production and infection progression in infected trophoblast cultures. HCMV is sensitive to low pH (Kempf *et al.*, 1991) and treatment of infected cells with a low pH citrate inactivation buffer has been reported to inactivate any externally associated nonpenetrated virus (Compton, 2000). Following the 24-hour challenge period, trophoblast cultures were washed extensively with medium (IMDM) or treated with saline at a low pH for two minutes and then washed (low pH). The culture supernatants and cell lysates were analyzed for infectious virus 48 hours later (Table 6.1). Alternatively, inoculated cultures were washed extensively after the 24-hour challenge period and cultured for 48 hours in the presence of intravenous immune globulin (IVIG), which contains neutralizing antibodies to HCMV (Table 6.1; Tenold, 1983; Zaia, 1993). Neither of these treatments altered the number of nuclei per well at either time point when compared to the IMDM group (Figure 6.1). Although the frequency of IE-positive nuclei was slightly different in this experiment between treatments in the –EGF cultures at two days after viral challenge,

differences were not reproducible and none were observed five days later. There were no differences in infection levels of +EGF cultures at either time point (Table 6.1).

Confirmation of residual inoculum and its effective removal by these methods was demonstrated by the lack of infectious virus detected in the supernatants or cell lysates of low pH or IVIG treated cultures when compared to those washed in medium alone at the early time point (Table 6.1). Virus detected in the cell lysates of IMDM-washed cultures two days after challenge was unlikely to be progeny virus for reasons stated above but was probably due to externally adherent virus. This adherent virus was removable or neutralized by the low pH or IVIG treatments. However, the presence of residual inoculum did not affect the progression of infection as measured by detection of IE antigen or amounts of progeny virus produced eight days after viral challenge. The only difference detected was at day 8 in –EGF cultures between the IMDM and IVIG groups in cell-associated progeny virus levels, which may have been a result of IVIG carryover during cell lysate preparation. In all further experiments with trophoblasts a low pH wash directly after the 24-hour inoculation period as well as just prior to analysis of cell-associated progeny virus was done to remove any externally adherent virus.

6.2.2 Rapid progression and higher progeny virus production in infected –EGF compared to +EGF cultures

While investigating the phenomenon of adherent virus, it was observed that infected –EGF cultures produced more progeny virus than infected +EGF cultures (Day 8; Table 6.1). This observation was confirmed in six independent studies that examined progression, kinetics and progeny virus production in infected +EGF and –EGF cultures. A sample experiment chosen specifically because +EGF and –EGF cultures were initially infected at the same frequency confirmed that differences between the two cultures were not simply because of different initial infection levels (Figure 6.2). The frequency of IE-positive nuclei increased more rapidly in infected –EGF cultures than in infected +EGF cultures (Figure 6.2A). The majority of cells stained strongly positive for pp65 in the –EGF cultures whereas <10% were detected in +EGF cultures by 20 days after viral challenge (Figure 6.3A and 6.3B). Characteristic cytopathic manifestations of HCMV infection such as enlarged cells with nuclear inclusions (Jesionek, Kiolemenoglou, 1904;

Ribbert, 1904; Hanshaw, 1968) were observed in all infected trophoblast cultures but more predominantly in –EGF cultures (e.g. enlarged inset picture, Figure 6.3B).

Progeny virus production, as predicted by the pp65 expression levels, was higher in infected –EGF cultures than in infected +EGF cultures at all time points (Figure 6.2B and 6.2C). To analyze progression of infection, the total cell-associated PFU produced per well was compared to the total number of IE-positive cells per well in four experiments performed in a similar manner. Although no differences were observed three days after viral challenge, by 11 days the amount of virus produced was significantly higher in –EGF cultures than in +EGF cultures ($p=0.002$ using a repeated measures analysis; Figure 6.4). As well the amount of progeny virus being produced by each IE-positive cell in –EGF cultures was greater than in +EGF cultures. Using a one factor repeated measures analysis this difference was marginally significant with a p value of 0.086. The observed power of the experiment was only 42% and more trophoblast preparations would have to be assessed in order to make any strong conclusions.

These results were not exclusive to a laboratory-adapted strain of HCMV since a clinical isolate, Kp7, showed a similar trend (Table 6.3). More progeny virus was produced in Kp7 infected –EGF cultures resulting in higher amounts of cell-associated progeny virus being produced per IE-positive nucleus than in +EGF cultures even when the frequency of IE-positive nuclei in –EGF cultures was lower than in +EGF cultures.

6.2.3 Increased cell loss in infected –EGF cultures

In four out of five experiments performed, increased cell loss as a result of HCMV infection was observed in –EGF cultures compared to +EGF cultures (Figures 6.3 and 6.5). Using a repeated measures analysis of variance, a marginally significant difference ($p = 0.057$) between +EGF and –EGF trophoblast cultures regardless of time after challenge was found. In other words –EGF cultures lost significantly more cells over the entire time period than +EGF cultures. Significantly more cells were lost after six days post-challenge than before regardless of culture type ($p = 0.027$). There was no significant interaction between time and culture type ($p = 0.155$). The power of this analysis was only 26% and confirmation of the results would require repetition with a larger sample size, however, a trend towards enhanced cell loss in infected –EGF cultures

has been clearly found. To determine if cell loss in –EGF cultures was occurring by apoptosis, TUNEL was performed on infected –EGF cultures at various time points (Figure 6.6). Increasing cell loss was associated with increasing TUNEL-positive nuclei suggesting that apoptosis may account for at least some of the cell loss.

In one of five experiments performed an exceptional loss of cells occurred in infected +EGF cultures but not –EGF cultures 11 days after viral challenge (Figure 6.7). Infection progression and progeny virus production in this experiment were similar to the other four experiments in that –EGF cultures produced more virus per IE-positive cell and infection levels increased more rapidly over time than in +EGF cultures (e.g. Figure 6.2). Cell loss was again associated with an increase in TUNEL-positive nuclei in the infected +EGF culture, suggesting that apoptosis may contribute to cell loss.

6.2.4 Optimal trophoblast HCMV infection remains inefficient compared to fibroblast infection

Using optimal challenge protocols, infection of trophoblasts was still less efficient and progressed at a slower rate than infection of fibroblasts (compare Figure 6.2 to Table 6.3). After 12 days of infection, fibroblasts challenged with an MOI of 0.005 for only two hours progressed to 100% IE-positive nuclei with an average of 1390 cell-associated virions produced per IE-positive nucleus. Infection in –EGF trophoblast cultures initially challenged with an MOI of 10 for 24 hours progressed to only 40% IE-positive nuclei with an average of 55 cell-associated virions produced per IE-positive nucleus by 12 days of infection. Cell loss was also more pronounced in infected fibroblast cultures than trophoblast cultures: 84.1% compared to 48.0% respectively, with the exception of the experiment depicted in Figure 6.7 (65.4%).

6.2.5 EGF does not reduce infection progression or progeny virus production

Treatment of fibroblasts with EGF 24 hours prior to viral challenge has been reported to decrease progeny virus production (Knox *et al.*, 1978). To investigate whether the presence of EGF decreased infection progression or progeny virus production, trophoblasts were first cultured in the absence of EGF for 24 hours and then challenged in and subsequently cultured with three concentrations of EGF or without EGF. Infection

was assessed seven days after viral challenge by staining for IE and pp65 antigens and determining progeny virus production (Figure 6.8). EGF did not reduce infection levels or progeny virus production at any concentration tested. In the experiment depicted these cultures demonstrated increased infection levels relative to cultures challenged and incubated without EGF. In a second experiment there was no difference detected between any of the groups. Thus the presence of EGF itself does not explain the reduced infection levels observed in +EGF cultures, which again points to the importance of the maturation state of the culture at the time of viral challenge.

6.3 SUMMARY OF RESULTS

6.3.1 Optimization of viral challenge protocols in trophoblasts led to the observation of residual inoculum initially adherent but eventually released into culture medium. A low pH wash successfully removed adherent virus.

6.3.2 IE and pp65 antigen frequencies increased more rapidly with higher progeny virus production in infected –EGF trophoblast cultures than in +EGF cultures even when initial infection efficiencies were identical.

6.3.3 In most cases both cell loss and apoptosis increased over time to a greater extent after viral challenge in infected –EGF cultures compared to infected +EGF cultures.

6.3.4 Even using optimal viral challenge protocols to infect trophoblast cultures, the infections were still less efficient than those observed in fibroblast cultures. The majority of trophoblasts remained resistant.

6.3.5 The reduced IE, pp65 and progeny virus production observed in infected +EGF cultures was independent of the presence of EGF itself.

Table 6.1[a] Residual adherent viral inoculum and removal with low pH or IVIG treatment

Treatment after HCMV challenge	Days after HCMV challenge	% IE-positive nuclei[b]		Virus in supernatant (/mL)[c]		Cell-associated virus (/mL)[c]	
		+EGF	-EGF	+EGF	-EGF	+EGF	-EGF
IMDM[d]	2	2.6 ± 0.5	9.3 ± 0.6	24600 ± 4703	8656 ± 1228	10933 ± 2003	11133 ± 2468
	8	4.3 ± 0.4	13.0 ± 1.6	360 ± 58	3937 ± 1348	36667 ± 7656	560000 ± 213979
pH 3.0[e]	2	2.4 ± 0.3	12.2 ± 0.8	62.0 ± 15.6	8.9 ± 7.7	156 ± 113	213 ± 40.0
	8	3.6 ± 1.1	14.9 ± 1.7	244 ± 252	2204 ± 608	31067 ± 7311	324667 ± 85049
IVIG[f]	2	1.9 ± 0.2	7.5 ± 0.8	0	0	115 ± 88.6	98 ± 78
	8	3.7 ± 0.3	13.2 ± 2.3	2497 ± 764	2986 ± 353	40200 ± 22525	193667 ± 36199

a Results are representative of two independent experiments.

b +EGF and –EGF trophoblast cultures were challenged with AD169 for 24 hrs at an MOI of 10, treated according to d, e and f and stained for HCMV IE antigen at either 2 or 8 days after viral challenge. Percentages were calculated as the mean ± SD in triplicate wells.

c Trophoblasts were challenged and cultured as in (b). Supernatants were removed at either 2 or 8 days after viral challenge, the cultures washed five times in IMDM and cell lysates prepared. The mean ± SD of infectious virions per mL in triplicate wells of supernatant or cell lysates was determined.

d After a 24-hr challenge, cultures were washed five times with 2% FBS/IMDM and cultured a further 2 or 8 days in 2% FBS/IMDM.

e After a 24-hr challenge, cultures were treated with saline at a pH of 3.0 for 2 min, washed five times in IMDM and cultured a further 2 or 8 days in 2% FBS/IMDM.

f After a 24-hr challenge, cultures were washed five times with 2% FBS/IMDM and cultured a further 48 hr with IVIG at 10 mg/mL in 2% FBS/IMDM. After 48 hr IVIG was removed and the cultures assessed or further cultured for 6 days in 2% FBS/IMDM.

Table 6.2[a] Residual viral inoculum in infected HEL cultures

	% IE-positive nuclei[a]			Residual virus in culture supernatants (PFU/mL)[e]		
	IMDM[b]	pH 3.0[c]	IVIG[d]	IMDM[b]	pH 3.0[c]	IVIG[d]
2 hr viral challenge	8.00 ± 0.30	10.7 ± 0.38	8.00 ± 1.36	13.3 ± 23.1	0	4.43 ± 7.68
24 hr viral challenge	51.6 ± 3.75	58.1 ± 3.07	49.1 ± 1.53	8.87 ± 7.68	13.3 ± 23.1	88.9 ± 27.7

a Confluent HEL cultures were challenged with AD169 for 2 or 24 hrs at an MOI of 0.2, treated according to b, c or d, further incubated for 20 hours and stained for HCMV IE antigen. Percentages were calculated as the mean ± SD in triplicate wells. The experiment has also been performed at an MOI of 0.1 with similar results.

b After viral challenge, cultures were washed five times with 2% FBS/IMDM and cultured a further 20 hrs in 2% FBS/IMDM.

c After viral challenge, cultures were treated with saline at a pH of 3.0 for 2 min, washed five times in IMDM and cultured a further 20 hrs in 2% FBS/IMDM.

d After viral challenge, cultures were washed five times with 2% FBS/IMDM and cultured a further 20 hrs with IVIG at 10 mg/mL in 2% FBS/IMDM.

e Supernatants were removed prior to fixation for staining and the mean ± SD of infectious virions (PFU) per mL in triplicate wells determined.

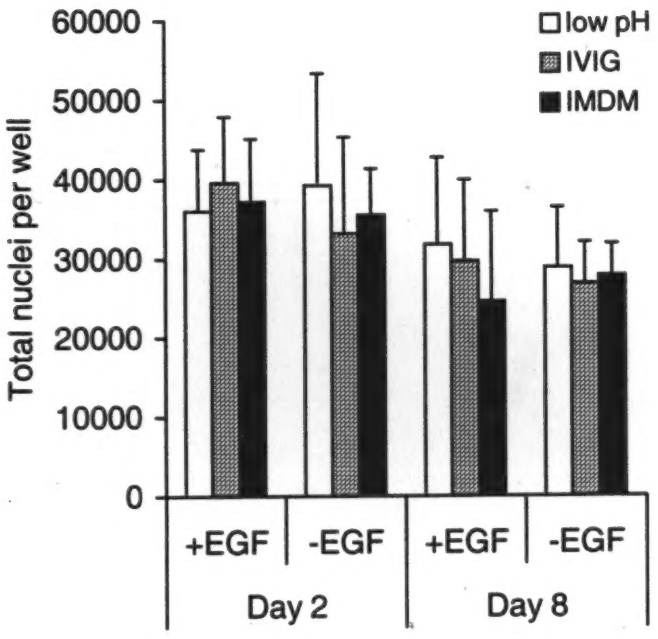


Figure 6.1 Loss of nuclei in infected trophoblast cultures from Table 6.1.
+EGF and –EGF cultures were challenged with AD169 and treated according to Table 6.1. Cultures were fixed and counterstained with hematoxylin and the total number of nuclei estimated by taking the average number in 5 fields and multiplying by the total number of fields per well. Each point represents the average ± SD of triplicate wells.

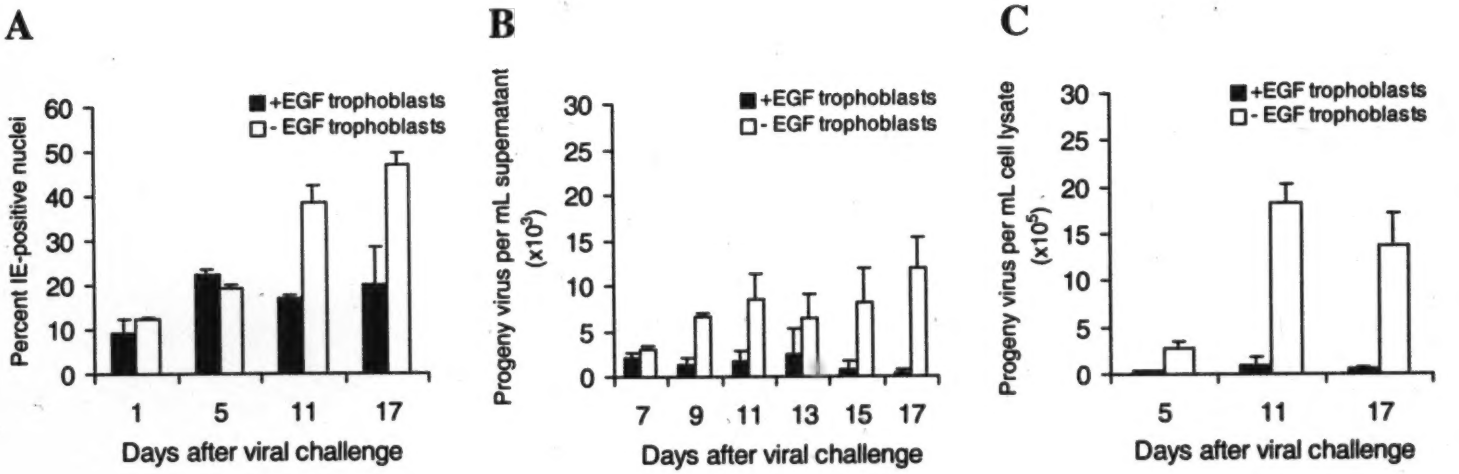


Figure 6.2 Progression of HCMV infection in +EGF and –EGF trophoblast cultures.
Trophoblasts incubated with and without EGF were challenged with AD169 at an MOI of 10 for 24 hrs, treated with low pH saline and further cultured for the time points indicated. **(A)** Cultures were stained for IE antigen and the mean percentage ± SD of triplicate wells determined. Supernatants were removed **(B)** and cell lysates prepared **(C)** and each assayed for infectious virus at the indicated times. Virus titres are depicted as ($x10^3$) for supernatants **(B)** and ($x10^5$) for cell lysates **(C)**. Each bar represents the mean ± SD of triplicate wells in one out of six experiments performed.

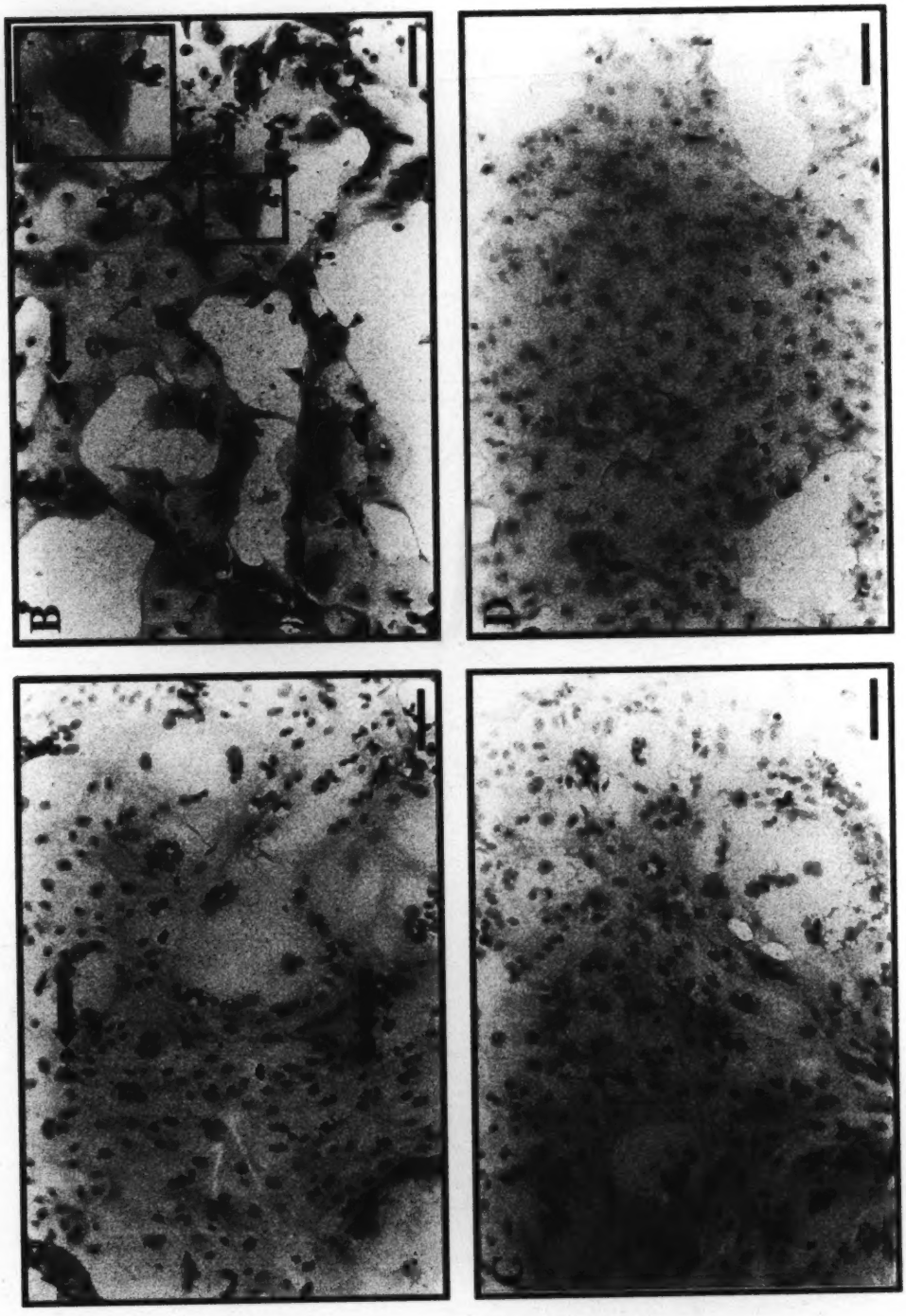

B
D

Figure 6.3 pp65 antigen expression in HCMV infected +EGF and –EGF cultures. Trophoblasts were cultured with EGF for five days **(A,C)** or without EGF for 1 day **(B,D)** and challenged with AD169 at an MOI of 10 for 24 hours **(A,B)** or left uninfected **(C,D)**. At 20 days after viral challenge, the cultures were immunohistochemically stained for pp65 antigen. The solid black arrows denote pp65-positive nuclei. The inset in (B) denotes typical nuclear changes in HCMV infected cells. Photographs are representative of five experiments performed. Bar, 50 μm.

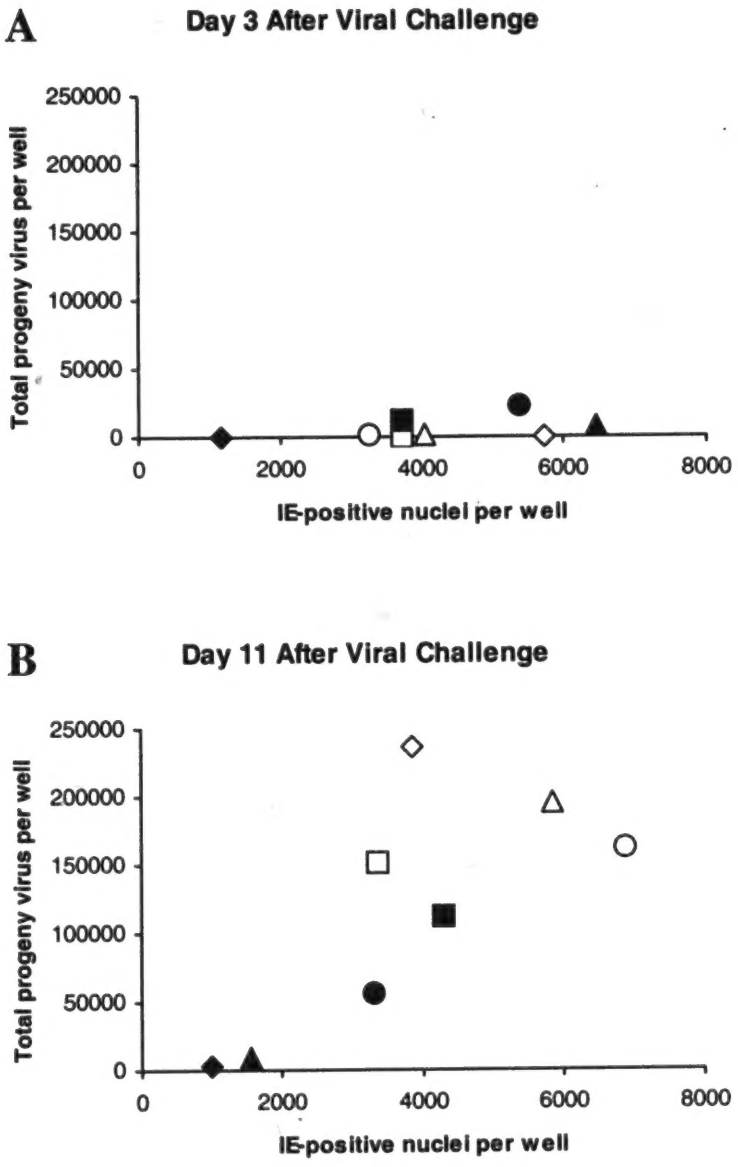


Figure 6.4 The relationship of cell-associated PFU to IE-positive nuclei per well in +EGF and –EGF trophoblast cultures.

The total number of IE-positive nuclei per well was graphed against the respective total cell-associated infectious progeny virus (PFU) produced in that well Day 3 after viral challenge **(A)** and Day 11 after viral challenge **(B)**. Solid symbols represent +EGF trophoblast cultures and open symbols represent –EGF trophoblast cultures. A total of four independent experiments are depicted by different symbols. See text for details of statistical analysis using a one factor repeated measures analysis as performed by Damon Mayes.

Table 6.3[a] Infection in +EGF and -EGF trophoblast cultures with an HCMV clinical isolate, Kp7

	% IE-positive nuclei[c]		Cell-associated progeny virus (/mL)[d]		Cell-associated progeny virus per IE-positive nucleus[e]	
	+EGF	-EGF	+EGF	-EGF	+EGF	-EGF
Day 7[b]	10.9 ± 2.06	2.8 ± 0.56	$5.34 \times 10^4 \pm 1.61 \times 10^4$	$5.25 \times 10^5 \pm 2.37 \times 10^4$	3.0	152
Day 11	18.6 ± 2.80	7.8 ± 1.3	$1.25 \times 10^5 \pm 1.06 \times 10^5$	$1.71 \times 10^6 \pm 9.62 \times 10^4$	6.8	187

a Results are representative of two independent experiments.

b Days after viral challenge.

c +EGF and -EGF trophoblast cultures were challenged with a clinical isolate (Kp7) at an MOI of 3.0 for 24 hours, treated with low pH, washed and cultured an additional 7 or 11 days and then stained for HCMV IE antigen. Percentages were calculated as the mean ± SD of triplicate wells.

d Values are reported as the mean ± SD in triplicate wells of infectious virions per mL of cell lysate.

e The average number of cell-associated progeny virus produced per well was divided by the average number of IE-positive nuclei in each well.

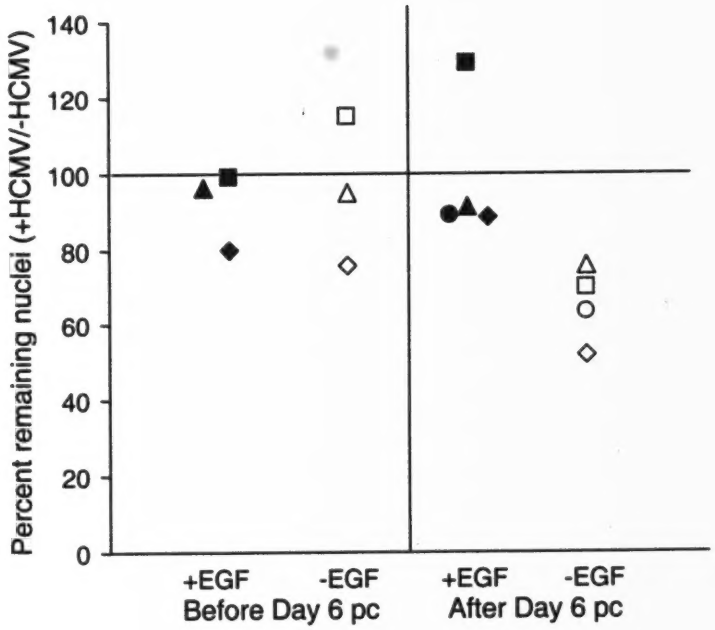


Figure 6.5 Loss of nuclei in infected and uninfected trophoblast cultures.
Infected and uninfected trophoblast cultures were counterstained with hematoxylin at the indicated time points. The total number of nuclei present per well was estimated as described in Figure 6.1. The number of nuclei in infected wells were compared to nuclei in uninfected wells and the percent remaining nuclei determined as follows:

$$\frac{\text{\# nuclei in infected cultures} \times 100}{\text{\# nuclei in uninfected cultures}}$$

Solid symbols represent +EGF cultures (n=3). Open symbols represent –EGF cultures (n=4). Independent experiments are represented by the four different symbols. See text for details of statistical analysis using repeated measures analysis of variance as performed by Damon Mayes.

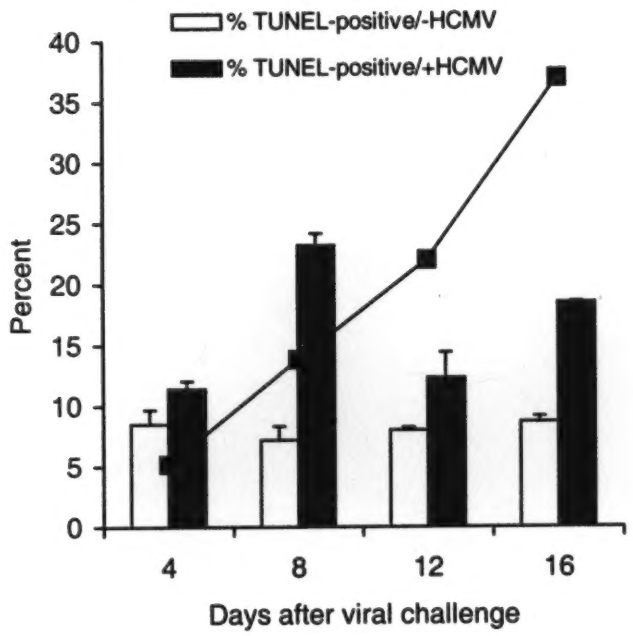


Figure 6.6 Comparison of % TUNEL-positive nuclei and % nuclei loss in infected and uninfected –EGF trophoblast cultures.

-EGF trophoblast cultures were challenged with AD169 at an MOI of 10 for 24 hrs or mock infected and percent TUNEL-positive nuclei determined in each culture (bars). The total number of nuclei per well was estimated as described in Figure 6.1 and percent nuclei loss (■) calculated by:

$$100 - \frac{\text{\# nuclei in infected cultures} \times 100}{\text{\# nuclei in uninfected cultures}}$$

Where error bars are not seen in the bar graphs, error was less than the width of the marker or zero. Results are representative of two independent experiments performed.

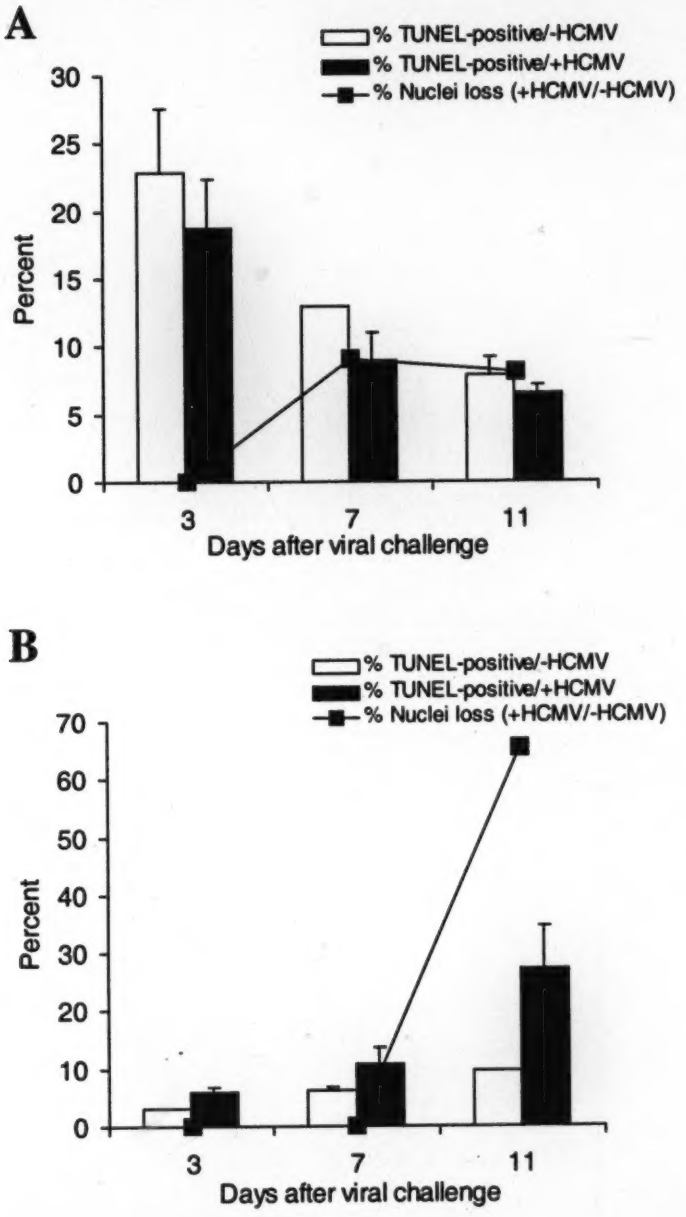


Figure 6.7 One experiment in five showed increased loss of nuclei and TUNEL-positive nuclei in infected +EGF cultures and not –EGF cultures.
-EGF **(A)** and +EGF **(B)** trophoblast cultures were challenged with AD169 at an MOI of 10 for 24 hrs or mock infected and percent TUNEL-positive nuclei determined in each culture (bars). Percent nuclei loss (■) was calculated as described in Figure 6.6. Where error bars are not seen in the bar graphs, error was less than the width of the marker or zero.

Table 6.4[a] HCMV infection of HEL cells

Days after challenge[b]	% IE-positive nuclei[c]	Progeny virus in supernatant (/mL)[d]	Cell-associated progeny virus (/mL)[d]	Cell loss[e]
Day 4	1.5 ± 0.36	$1.03 \times 10^2 \pm 4.50 \times 10^1$	$1.09 \times 10^3 \pm 3.84 \times 10^2$	0
Day 8	53.8 ± 5.0	$4.90 \times 10^5 \pm 2.37 \times 10^5$	$1.22 \times 10^6 \pm 7.13 \times 10^5$	$10.0 \pm 3.1\%$
Day 12	100 ± 0	$2.48 \times 10^7 \pm 6.08 \times 10^6$	$6.14 \times 10^6 \pm 1.51 \times 10^5$	$84.1 \pm 15.2\%$

a Results are representative of two independent experiments.
b Human embryonic lung fibroblasts (HEL) challenged with AD169 for two hrs at an MOI of 0.005 and cultured for a further 4, 8 or 12 days.
c Percentages were calculated as the mean ± SD in triplicate wells.
d Values are reported as the mean ± SD in triplicate wells of infectious virions per mL of culture supernatant or cell lysate.
e Percent cell loss (+HCMV/-HCMV) determined as described in the legend for Figure 6.6.

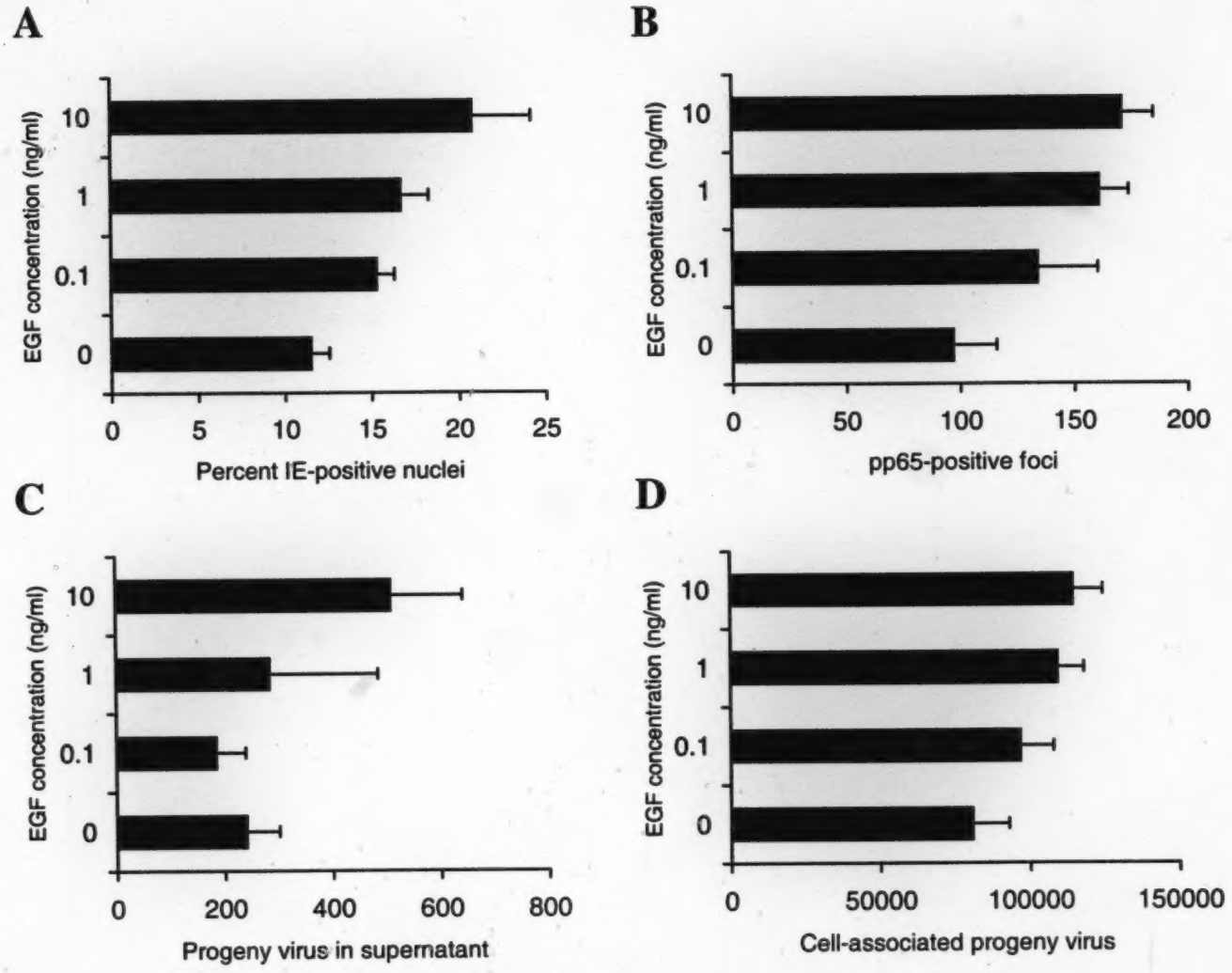

A
EGF concentration (ng/ml)
10
1
0.1
0
0
5
10
15
20
25
Percent IE-positive nuclei
B
EGF concentration (ng/ml)
10
1
0.1
0
0
50
100
150
200
pp65-positive foci
C
EGF concentration (ng/ml)
10
1
0.1
0
0
200
400
600
800
Progeny virus in supernatant
D
EGF concentration (ng/ml)
10
1
0.1
0
0
50000
100000
150000
Cell-associated progeny virus

Figure 6.8 Effects of EGF on infection progression and progeny virus production in –EGF trophoblast cultures.

After 24 hrs of culture –EGF cultures were challenged with AD169 at an MOI of 10 in the absence or presence of 10 ng/mL, 1 ng/mL or 0.1 ng/mL of EGF for 24 hrs. After a low pH treatment and extensive washing, cultures were maintained in the same concentrations of EGF as during viral challenge for a further 7 days with medium changes every 48 hours. Immunohistochemical detection of IE **(A)** and pp65 **(B)** antigens and progeny virus titres in culture supernatants **(C)** or cell lysates **(D)** was assessed. Each bar represents the average of triplicates ± SD in one of two experiments performed.

CHAPTER 7.0 DEVELOPMENT OF A TWO-SIDED TROPHOBLAST CULTURE MODEL ON SEMI-PERMEABLE MEMBRANES

(The majority of data presented in this chapter was published in *Placenta* 22:70-79, 2001 and entitled "Villous trophoblasts cultured on semi-permeable membranes form an effective barrier to passage of high and low molecular weight particles," by D.G. Hemmings, B. Lowen, R. Sherburne, G. Sawicki and L. J. Guilbert. **Note: Figures 7.8 and 7.9 were performed by parties other than me and are presented for the sake of completeness.)**

7.1 INTRODUCTION

I have shown that trophoblasts isolated from the chorionic villi of first trimester or term placentas could be permissively infected with HCMV. Progeny virus remained predominantly cell-associated with little apical release in a culture model where only interactions with the apical surface (maternal) of the trophoblasts could be detected. Trophoblasts are fetally derived epithelial cells with tight junctions and distinct apical and basolateral surfaces. A crucial and more physiologic assessment of the effects of a productive HCMV infection in trophoblasts would be to determine if infectious virus is released from the basal surface since, *in vivo*, this is the direction virus must pass to reach the fetus. Development of a model to examine this possibility is the subject matter of this chapter.

The barrier functions of the human villous placenta must be investigated *in vitro* using cell culture or intact placental perfusion models. Perfusion of placental tissues provides an accurate *ex vivo* model of substance movement from the maternal to the fetal side of the ST (Omarini *et al.*, 1992; Muhlemann *et al.*, 1995; Polliotti *et al.*, 1996). However, since cells in perfused placentas remain viable for < 48 hours, this model is not suitable for studies investigating transmission by infection with a virus such as cytomegalovirus (Halwachs-Baumann *et al.*, 1998). Culture of the choriocarcinoma cell line BeWo (Pattillo, Gey, 1968) on semipermeable membranes, which allows access to both apical and basal cell compartments, produces tight-junctioned monolayers (Cerneus,

syncytium in this culture results in large cell-free culture gaps (Liu *et al.*, 1997), making them unsuitable for studies of barrier function, especially those involving infectious pathogens. Further, these cells are transformed, complicating interpretation of experiments involving infection and polarized release (Ringler, Strauss, 1990).

Several models of primary villous trophoblast cultured on semipermeable substrata have been established. Cultures of a primary preparation on porous membranes displayed enhanced differentiated trophoblast characteristics although culture continuity was not documented (Truman, Ford, 1986). In the amniotic membrane model used in studies of glucose transport (Bullen *et al.*, 1990), it is unclear if villous syncytia are formed on the relatively thick substrata. An artificial filter membrane model used in studies of immunoglobulin transcytosis (Sooranna, Contractor, 1991) provides a thinner model of the TBM with a regulated pore size. In the latter two models, a significant fraction of transport was trophoblast mediated but there was considerable (up to 70% of the total) non-saturable transport suggesting disruption of tight junctions or the presence of gaps between trophoblast syncytia (Bloxam *et al.*, 1997b). Culture discontinuity is a serious technical impediment for studies of barrier function.

There are a number of obstacles in the development of confluent artificial membrane cultures using primary villous trophoblasts: first, neither +EGF nor –EGF treated trophoblasts proliferate in culture (Contractor, Sooranna, 1982; Kliman *et al.*, 1986; Aplin, 1991; Yui *et al.*, 1994; Garcia-Lloret *et al.*, 1996) leaving gaps between cells or syncytia; second, plating higher numbers of cells to fill in the gaps results in self-association into non-adhering floating aggregates (unpublished observations; Lowen, B., Guilbert, L.J.); third, contamination of strongly adhering placental macrophages disrupt trophoblasts in culture, perhaps from local release of TNFα which stimulates trophoblast apoptosis (Yui *et al.*, 1994; Garcia-Lloret *et al.*, 2000); and finally, even a very low (0.1%) contamination of fibroblasts disrupts epithelial tight junctions via colony formation after a week in culture (Vettenranta *et al.*, 1986; Yui *et al.*, 1994).

This chapter describes the development of effective confluent barrier cultures of trophoblasts grown on semi-permeable membranes that allow access to both apical and basolateral surfaces. The highly purified (>99.99%) and viable long-term cultures (>3 weeks with no fibroblast colonies) of non-EGF treated trophoblasts that differentiate in

the presence of EGF (Morrish *et al.*, 1987; Garcia-Lloret *et al.*, 1996; Kilani *et al.*, 1997; Morrish *et al.*, 1997) that are used in the Guilbert laboratory surmount the majority of issues raised above. Using these trophoblast cultures and minimizing culture gaps through multiple seedings interspersed with incubations in EGF, effective long-term barrier cultures of differentiated trophoblasts grown on semi-permeable insert membranes were developed. Using light and electron microscopy, and measurements of transepithelial electrical resistance, transepithelial diffusion of high (^{14}C-dextran and human cytomegalovirus) and low (^{3}H-inulin) molecular weight substances and directional secretion of the matrix metalloproteinase MMP-9, these cultures are shown to consist of syncytialized, tight-junctioned, confluent and functionally polarized trophoblast layers.

7.2 RESULTS

7.2.1 HCMV diffuses through 3.0 μm pores of cell-free insert membranes

HCMV is the largest virus in the *Herpesviridae* family with a diameter of approximately 200 nm (reviewed in Mocarski, 1996), so confirmation of diffusion through insert pores was essential. No virus was detected in the basal chamber in initial experiments using cell-free insert membranes with 0.45 μm pores after two hours. However, in a representative experiment virus diffused through membranes with 3.0 μm pores at an initial rate of 10.9% per hour (Figure 7.1). This rate plateaus by 4 hours to approximately 14% likely because of absorption and/or heat inactivation of the virus (see Figure 5.2B; Vonka, Benyeshmelnick, 1966). The average rate of diffusion in three experiments is presented in Table 7.1. All experiments to develop the culture model and investigate viral passage through inserts were therefore done using 3.0 μm insert membranes.

7.2.2 Insert membrane cultures of Madin-Darby Canine Kidney (MDCK II) epithelial cells

As a comparative reference point for the barrier functions of trophoblast cultures, I cultured a standard tight-junctioned epithelial cell line, MDCK II (reviewed in Simons, Fuller, 1985), on semi-permeable fibronectin-coated 6.4 mm insert membranes with 3.0 μm pores. Light microscopy confirmed that these cultures were confluent (Figure 7.2A). A representative experiment demonstrated that MDCK II cells form an effective barrier to transepithelial diffusion of 2,000,000 mw ^{14}C-dextran (Figure 7.2B). In this experiment, the confluent MDCK II cells reduced initial transmembrane flux (the rate measured in the first 15 min) 184-fold compared to cell-free inserts. The actual initial transmembrane flux measurements averaged from three independent experiments are shown in Table 7.1. Transepithelial electrical resistance (TER) readings across the MDCK II cells cultured on the 6.4 mm inserts averaged 50.5 ± 18.5 ohms x cm^2, a value lower than previously reported values (100-250 ohms x cm^2) from cells cultured on larger (23.4 mm) inserts (Richardson *et al.*, 1981; Cerneus, van der Ende, 1991). However, my TER readings approached those of literature values when my MDCK II cells were cultured in 23.4 mm inserts (180 ohms x cm^2) suggesting a larger fraction of current

passing through the culture periphery in the smaller inserts.

7.2.3 Preparation of insert membrane cultures with term villous trophoblasts

Highly purified term villous CT were seeded at 2 x 10^5 cells per insert and cultured with EGF. Attempts to culture a single layer of trophoblasts to confluence gave variable results dependent on the trophoblast preparation used. The average TER varied from 0 to 40 ohms x cm^2 by day 12 of culture with holes and deterioration at the edges of the insert almost always detected by light microscopy. The lack of consistent results with one layer of cells along with the observed deterioration is why three cycles of three, four and five days each as detailed in the Materials and Methods were used to develop these cultures (see Figure 8.1 for a diagram of the model). The successive platings decreased the number and size of gaps between syncytial units (Figure 7.3A, B and C) until visual confluency was achieved after the third cycle of seeding by day 12 of culture (Figure 7.3C). Freshly seeded cells adhered preferentially to bare membrane and not to cells already attached and differentiated in response to EGF treatment (unpublished observations; Lowen, B., Guilbert, L.J). Immunohistochemical staining with anti-desmoplakin antibody, which marks tight-junctioned trophoblast cell boundaries (Douglas, King, 1990), showed day 14 insert membrane cultures to be a patchwork of syncytial sheets of varying sizes (Figure 7.4; Garcia-Lloret *et al.*, 1996).

7.2.4 Demonstration of an effective physical barrier in trophoblast insert membrane cultures

After each seeding of trophoblasts, TER and transepithelial diffusion of 2,000,000 mw ^{14}C-dextran and 5000 mw ^{3}H-inulin were measured. TER increased with each seeding of trophoblasts to an average of 59.7 ohms x cm^2 after the third plating on day 12 of culture (Table 7.1).

The diffusion rate of ^{14}C-dextran decreased with each seeding of trophoblasts. Figure 7.5 shows the diffusion time course from a representative experiment and Table 7.1 shows the average initial transepithelial fluxes calculated from a series of independent experiments. After the third seeding, diffusion rates through the trophoblast cultures compared favorably with that of confluent MDCK II cells (Table 7.1, compare Figures 7.2B and 7.5C). Diffusion through the MDCK II or triple-seeded trophoblast cultures

appeared to be passive since it was not competable by 100-fold excess of unlabelled 2,000,000 mw dextran (Figure 7.6). Transepithelial flux of an inert low molecular weight substance, ^{3}H-inulin, was also evaluated. Triple-seeded trophoblasts reduced transmembrane flux 18-fold to 1.91 ± 0.743 pmoles/hr/cm^2 (Table 7.1).

To further compare the barrier function of MDCK II cells and triple-seeded trophoblasts, 4 x 10^5 active HCMV particles were added to the insert membrane cultures. The number of virions passively diffusing through the cultures was monitored by placing the inserts in wells containing confluent HEL fibroblasts. The inserts were removed after two hours and the fibroblasts assessed for infection as outlined in the Materials and Methods. The transepithelial diffusion rate, determined by the number of infectious virus particles (PFU) diffusing in a two-hour period, was reduced 35-fold through 12-day triple-seeded trophoblast insert cultures from 5.69 ± 4.53 %/hr for cell-free inserts to 0.160 ± 0.219 %/hr. Intermediate values were observed after one or two seedings of trophoblasts (Table 7.1). No virus passed through confluent MDCK II cells over the time period analyzed.

7.2.5 Maintenance of an effective physical barrier in trophoblast membrane cultures

The low transepithelial flux rates observed after three seedings of trophoblasts and a total of 12 days of culture were maintained with slight improvements for both ^{3}H-inulin and ^{14}C-dextran after 32 days in culture (Table 7.1). Virus diffusion was reduced to < 1 PFU/2 hours when tested on 32-day triple-seeded trophoblasts and the average TER increased, suggesting a continuing maturation of these cultures over time. Long-term triple-seeded trophoblast cultures were confluent by light microscopy. An inverse correlation between ^{14}C-dextran transepithelial flux rates and TER was observed (Figure 7.7).

7.2.6 Ultrastructure of triple-seeded trophoblast insert membrane cultures

Desmoplakin staining and high TER measurements suggested the presence of tight junctions in triple-seeded trophoblast cultures. Tight junctions were demonstrated by the detection of characteristic electron dense desmosomes in transmission electron micrographs (Figure 7.8A). Multiple sheets of syncytium were found to overlap (Figure

7.8A, C) demonstrating that these cultures were not simple monolayers. Ten ultra-thin sections from six different areas of an insert membrane containing a 12-day triple-seeded trophoblast culture were evaluated by transmission electron microscopy for the numbers of cell layers (delineated by tight junctions and/or apposing plasma membranes). Two to three thin layers of overlapping syncytialized units were consistently observed over large areas of the membrane. An average thickness of 2.7 ± 0.20 cells was observed with only rare detection of areas with greater than three layers or thin one-cell layer syncytial sheets (e.g. Figure 7.8A).

In preliminary experiments, approximately 50% of the 3.0 μm pores in the semi-permeable membrane were observed by TEM to contain trophoblast pseudopods and these occasionally spread out to cover 14.8 ± 6.8% of the underside of the membrane. However, nuclei (which are much larger than the pores) were not observed on the underside of the membrane. An electron dense layer detected between the basal plasma membrane of the trophoblast and the insert membrane surface likely represents the TBM (see open arrow at the bottom left of Figure 7.8A).

7.2.7 Epithelial apical-basal orientation of triple-seeded trophoblast membrane cultures

Scanning electron micrographs showed somewhat short and stubby microvilli (Figure 7.8B) facing tissue culture medium, indicating a differentiated apical surface. However, denuded areas were also visible (Figure 7.8C).

The matrix metalloproteinase MMP-9 is released from epithelial and endothelial cells in a polarized fashion [apically from epithelial cells and basally from endothelial cells (Unemori *et al.*, 1990; Firth *et al.*, 1997)]. Since MMP-9 is secreted by cultured trophoblasts (Sawicki *et al.*, 2000), we asked whether confluent triple-seeded trophoblasts cultured on insert membranes were functionally polarized. As shown in Figure 7.9, >70% of MMP-9 released during a two-hour incubation period was in a basal direction, demonstrating functional polarity of these membrane cultures. The results also indicate that confluent triple-seeded trophoblasts cultured on insert membranes differ from other epithelial cells in their release pattern of MMP-9.

7.3 SUMMARY OF RESULTS

7.3.1 A confluent tight-junctioned trophoblast culture on semi-permeable membranes was developed by using a triple-seeding method interspersed with three to five day culture in EGF.

7.3.2 The triple-seeded trophoblast culture was tested by a number of parameters and compared favorably to MDCK II, a cell line often used in permeability studies.

a. The transepithelial electrical resistance measured across the cells was higher in trophoblast cultures than in MDCK II cultures.

b. The transepithelial diffusion rates of ^{14}C-dextran and HCMV were slightly higher for triple-seeded trophoblast cultures than for MDCK II cultures but still substantially less than the rates through cell-free inserts.

7.3.3 The triple-seeded trophoblast cultures maintained and generally improved on the above parameters as time in culture increased. An inverse relationship between transepithelial flux of ^{14}C-dextran and TER was shown.

7.3.4 Examination of these cultures by light and electron microscopy showed multiple layers of tight-junctioned multinucleated confluent cells with apical expression of microvillae and a possible trophoblast basement membrane between the cells and the insert membrane surface.

7.3.5 These triple-seeded trophoblast cultures showed directional release of MMP-9, demonstrating their functional polarity.

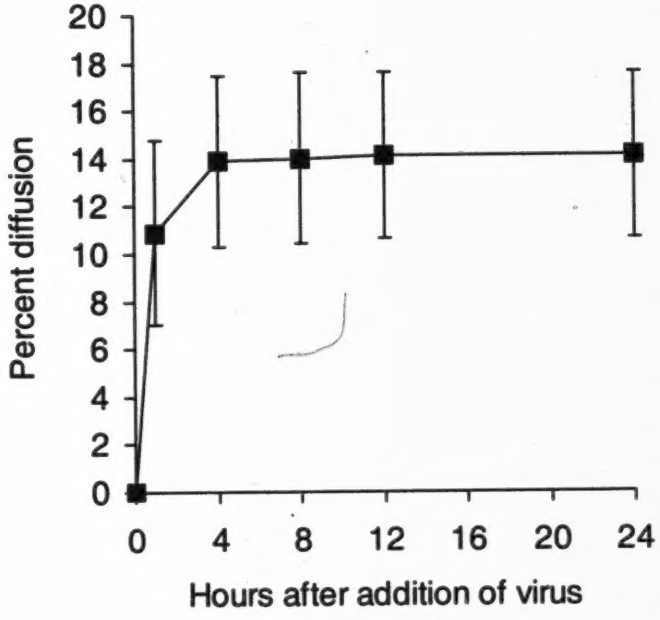


Figure 7.1 HCMV diffusion through insert membranes with 3.0 μm pores.
Insert membranes with 3.0 μm pores were presoaked with medium for 1 hr. After adding 4000 PFU of AD169, each insert was placed in a well containing confluent HEL cells and left for the time period indicated. The inserts were removed and the fibroblasts cultured for a further 18 hrs before assessment for IE expression. Each point represents the average ± SD of triplicate wells in one of three experiments performed.

Table 7.1 Comparison of MDCK II and trophoblasts cultured on 3.0 μm inserts.

	Cells	Culture Conditions	Transepithelial Electrical Resistance (ohms x cm^2)	HCMV Diffusion (%/hr)	^{14}C-Dextran Transepithelial Flux (pmoles/hr/cm^2)	^{3}H-Inulin Transepithelial Flux (pmoles/hr/cm^2)
1	None	Medium alone	0[a]	5.69 ± 4.53[c]	50.9 ± 37.8[d]	33.7 ± 22.4[e]
2	Trophoblasts	1 seeding, 3 days	1.2 (0.6-1.5)[b]	1.73 ± 2.25	23.7 ± 9.90	N/D
3	Trophoblasts	2 seedings, 7 days	2.7 (0.9-6.3)	1.14 ± 0.739	2.78 ± 0.467	N/D
4	Trophoblasts	3 seedings, 12 days	59.7 (19.2-99.6)	0.160 ± 0.219	0.858 ± 0.602	1.91 ± 0.743
5	Trophoblasts	3 seedings, 32 days	70.2 (34.5-141.3)	0.0 ± 0.0	0.484 ± 0.183	1.52 ± 0.333
6	MDCK II	Confluent culture	15.6 (3-30.3)	0.0 ± 0.0	0.286 ± 0.170	N/D

a Values for transepithelial electrical resistance (TER) are calculated by subtracting the average value for a cell-free insert (22.8). 1 (x=10), 2 and 3 (x=5), 4 (x=11), 5 (x=20) and 6 (x=8) where x = total number of inserts measured.

b Values reported as averages with the range of measurements in brackets.

c Values are the average ± SD of diffusion rates measured over two hours: 1 (n=3), 2, 3, 4 (n=2), 5 and 6 (n=1) where n = total number of experiments.

d Values are the average ± SD of transepithelial flux measured in the first 15 minutes: 1 (n=11), 2 and 3 (n=2), 4 (n=4), 5 (n=3) and 6 (n=2) where n = total number of experiments.

e Values as in d: 1 (n=7), 2 and 3 (N/D), 4 (n=3), 5 (n=1) and 6 (N/D) where n = total number of experiments.

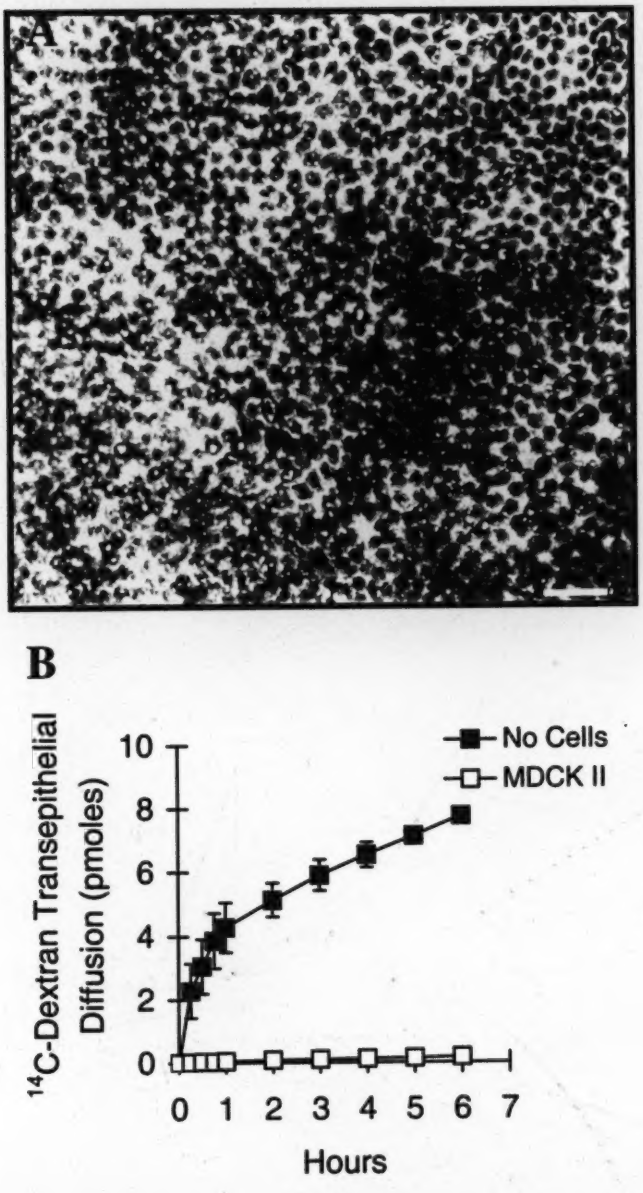


Figure 7.2 Transepithelial diffusion of high molecular weight ^{14}C-dextran through confluent MDCK II cells cultured on insert membranes.

(A) MDCK II cells grown to confluency on 3.0 μm insert membranes were fixed and stained with hematoxylin, Bar = 25 μm. **(B)** 12.4 pmole (85,000 dpm) of ^{14}C-dextran was added to each insert with and without confluent MDCK II cells and diffusion to the bottom chamber of a 24-well companion plate monitored as described in Materials and Methods. Results are expressed as the cumulative average diffusion in pmoles over time ± SD of four inserts from one of two independent experiments.

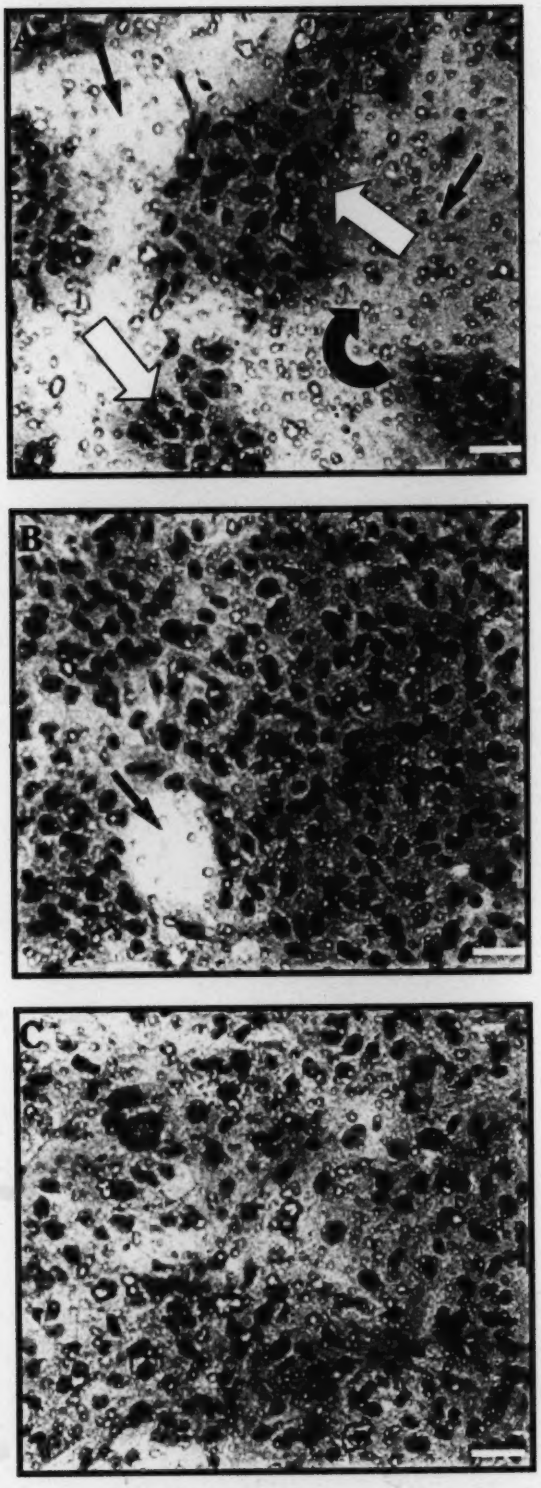

B
C

Figure 7.3 Light microscopy after each of three consecutive seedings of trophoblasts on insert membranes.

Trophoblasts were seeded sequentially onto 3.0 μm insert membranes, cultured in EGF, fixed and stained with hematoxylin and Stat stain after one **(A)**, two **(B)** or three **(C)** seedings, a total of three **(A)**, seven **(B)** or twelve **(C)** days in culture, as described in Materials and Methods. Straight black arrows denote cell-free areas of insert membrane; white arrows indicate cultured trophoblasts (all areas in Panel C without hematoxylin stained nuclei were positively stained for cytoplasm); the curved black arrow is an example of a 3.0 μm hole distorted by visualization through the standard microscope, Bar = 25 μm.

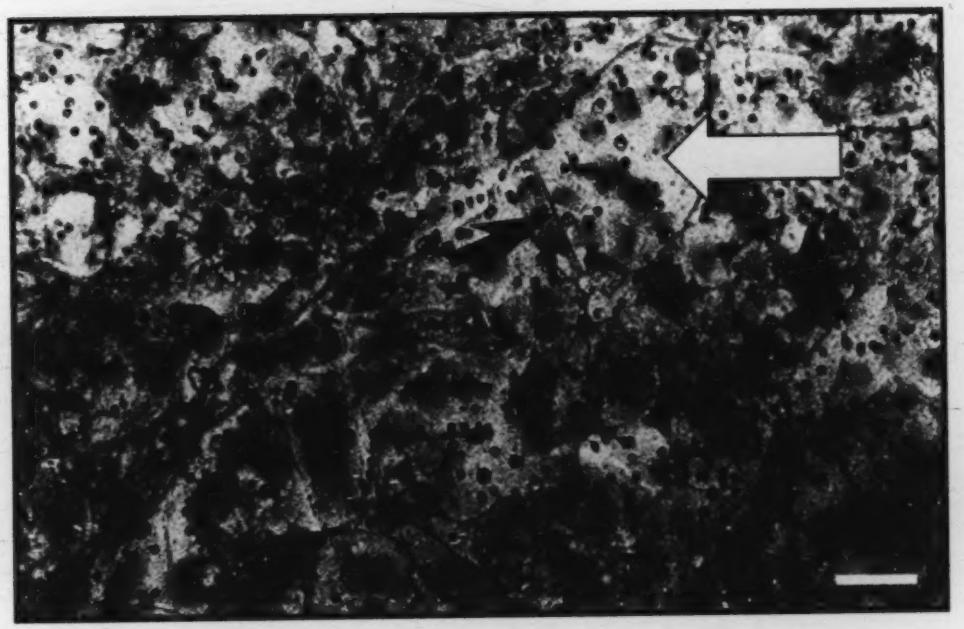
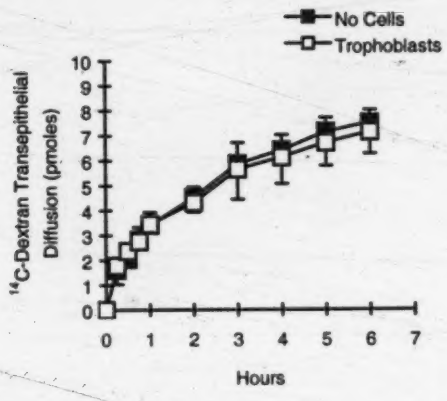
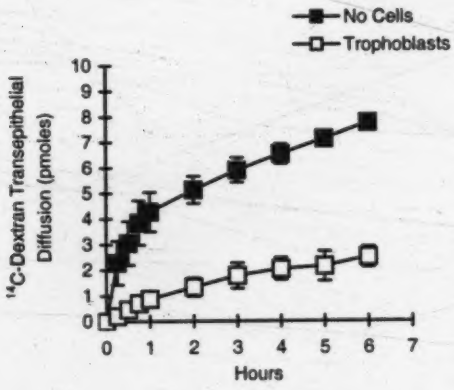


Figure 7.4 Desmoplakin staining of triple-seeded trophoblast insert cultures.
A 12-day triple-seeded trophoblast insert culture was fixed and stained with anti-desmoplakin antibody then counterstained with hematoxylin to visualize nuclei as described in the Materials and Methods. The black arrow indicates a desmoplakin-positive membrane and the white arrow denotes a multinucleated syncytial unit bordered by desmoplakin-positive membranes. Note the distorted 3.0 μm holes as denoted in Figure 7.3, Bar = 25 μm.

A


No Cells
Trophoblasts
^{14}C-Dextran Transepithelial Diffusion (pmoles)
0 1 2 3 4 5 6 7 8 9 10
0 1 2 3 4 5 6 7
Hours

B


No Cells
Trophoblasts
^{14}C-Dextran Transepithelial Diffusion (pmoles)
0 1 2 3 4 5 6 7 8 9 10
0 1 2 3 4 5 6 7
Hours

C

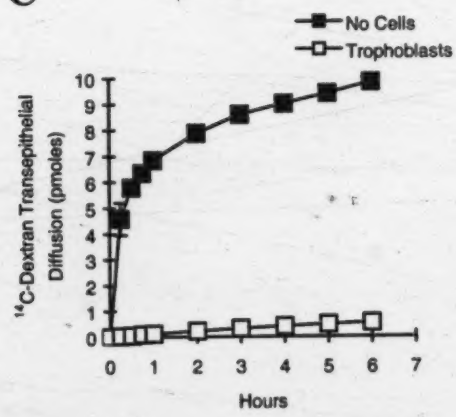

No Cells
Trophoblasts
^{14}C-Dextran Transepithelial Diffusion (pmoles)
0 1 2 3 4 5 6 7 8 9 10
0 1 2 3 4 5 6 7
Hours

Figure 7.5 Transepithelial diffusion of high molecular weight ^{14}C-dextran after consecutive seedings of trophoblasts on insert membranes.
Trophoblasts were seeded sequentially onto insert membranes and cultured in EGF as described in Materials and Methods. After one **(A)**, two **(B)** or three **(C)** seedings, a total of three **(A)**, seven **(B)** or twelve **(C)** days in culture, 12.4 pmole (85,000 dpm) of ^{14}C-dextran was added to each insert with and without cells and diffusion to the bottom chamber of a 24-well companion plate monitored as described in Materials and Methods. Results are expressed as the cumulative average diffusion in pmoles over time ± SD of two inserts from one of two **(A, B)** or four **(C)** independent experiments. Where error bars are not seen, error was less than the width of the marker or zero.

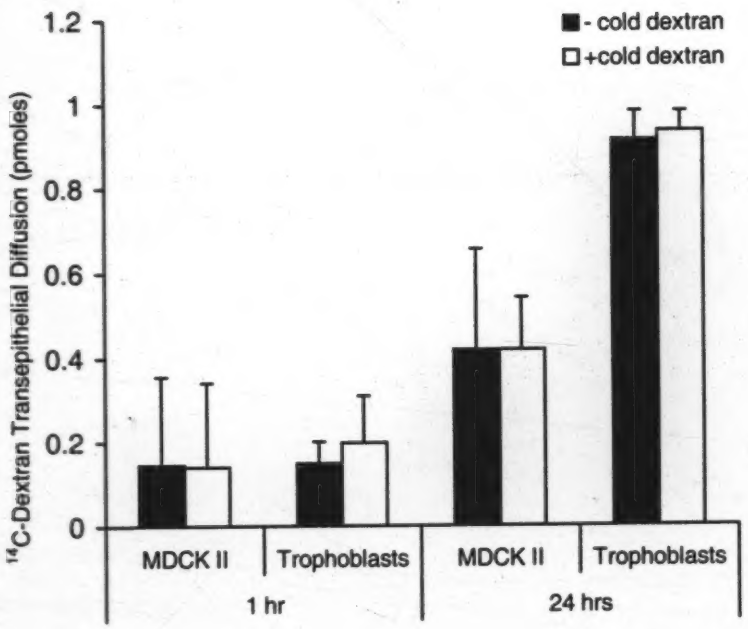


Figure 7.6 Excess cold high molecular weight dextran does not compete with ^{14}C-dextran diffusion.

MDCK II and triple-seeded trophoblast cultures were prepared as described in the legends to Figures 7.2 and 7.4. 12.4 pmole (85,000 dpm) of ^{14}C-dextran was added to each insert with or without a 100-fold excess of cold 2,000,000 mw dextran and diffusion to the bottom chamber of a 24-well companion plate monitored as described in Materials and Methods. Results are expressed as the cumulative average diffusion in pmoles over time ± SD of two independent experiments.

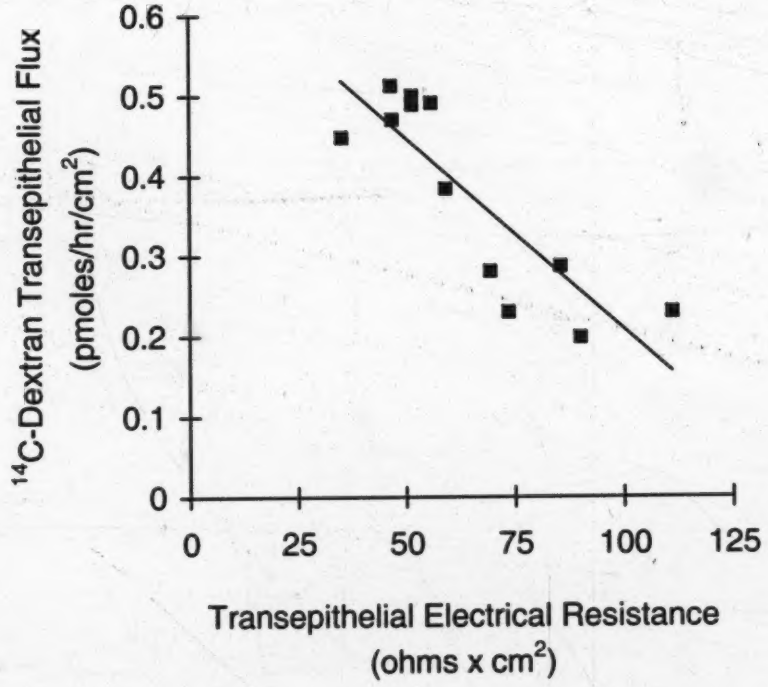


Figure 7.7 Transepithelial flux of ^{14}C-dextran and transepithelial electrical resistance (TER) are inversely related in 32-day trophoblast insert cultures.
The transepithelial flux of ^{14}C-dextran over six hours in pmoles/hr/cm^2 and TER in ohms x cm^2 were measured in 12 inserts with triple-seeded trophoblasts maintained in culture for 32 days after the first seeding of trophoblasts. The results were pooled from two independent experiments. A negative correlation was shown by linear regression analysis with a calculated regression coefficient $R = 0.875$ and a significance of $p < 0.005$.

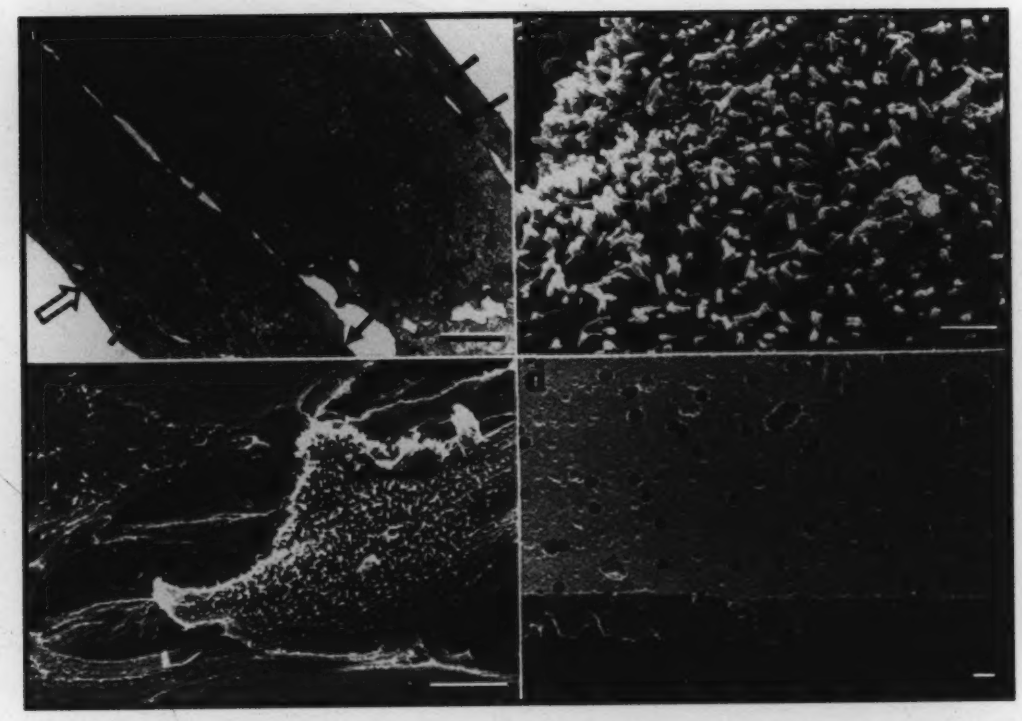


Figure 7.8 Electron microscopy (EM) of triple-seeded trophoblasts at day 25 of culture on insert membranes.
(a) Transmission EM showing four cell layers with tight junctions between cells (solid black arrows). The open arrow denotes an electron dense layer postulated to be the TBM. Bar = 1 μm. **(b)** Scanning EM showing details of microvillae. Bar = 5 μm. **(c)** Scanning EM showing microvilliated and denuded areas of trophoblasts with multiple cell layers. Bar = 5 μm. **(d)** Scanning EM of a cell-free membrane. Bar = 5 μm. EM procedures and photographs were performed by Dr. R. Sherburne (Department of Medical Microbiology and Immunology).

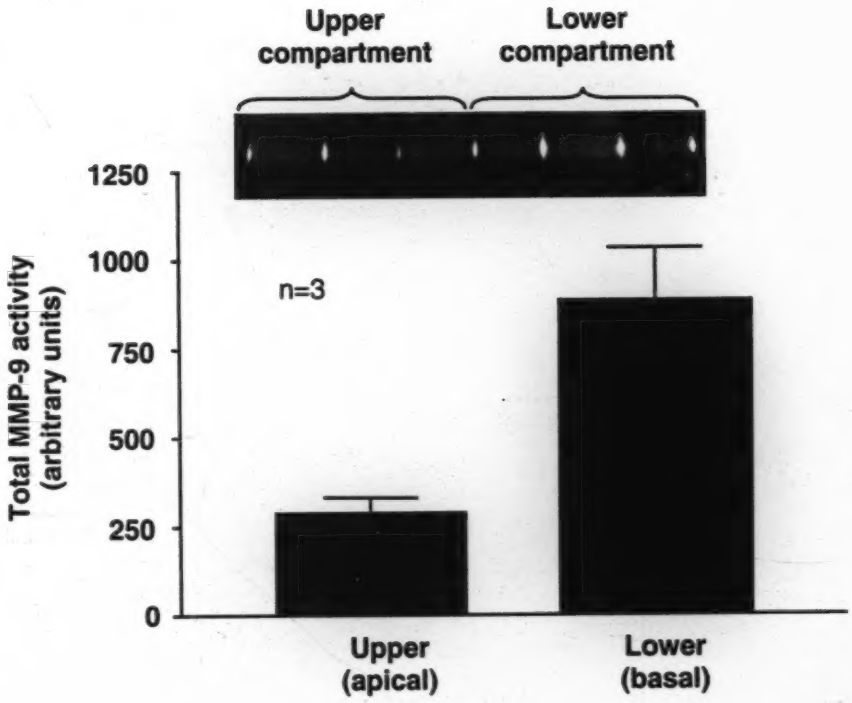


Figure 7.9 Polarized secretion of matrix metalloproteinase MMP-9 from insert cultures of triple-seeded trophoblasts.

Confluent cultures of triple-seeded trophoblasts were prepared on 3.0 μm pore insert membranes as described in the Methods. The medium was changed to serum-free IMDM and cultures incubated for a further 2 hrs. MMP-9 supernatant levels in both upper and lower culture chambers were assessed by zymography (the zymographs of three replicate cultures are shown in the upper part of the figure) and scan densities were normalized to reflect total MMP-9 release into the two chambers (see Methods). Depicted are the mean ± SD of three replicate cultures. These results have been repeated in an independent experiment using cells from a different placenta. All zymography was carried out by Dr. G. Sawicki (Department of Pharmacology).

CHAPTER 8.0 PROGENY VIRUS FROM INFECTED TROPHOBLASTS GROWN ON SEMI-PERMEABLE MEMBRANES REMAINS PREDOMINANTLY CELL-ASSOCIATED

8.0 INTRODUCTION

A number of polarized epithelial cells have been successfully cultured on permeable filters in order to investigate infectibility of apical versus basolateral surfaces, directional release of progeny and barrier function to various pathogens. Madin-Darby canine kidney (MDCK) epithelial cells have been used to show preferential basolateral infection by and release of vesicular stomatitis virus and preferential apical release of an avian influenza virus (Fuller *et al.*, 1984). The bacteria *Salmonella choleraesuis* was shown to move through and not between viable polarized MDCK cells (Finlay *et al.*, 1988) or a polarized colon epithelial cell line (Caco-2; Finlay, Falkow, 1990). The bacteria were protected in vacuoles and moved through the cells in a transcytosis-like manner. Another colonic cell line, T-84, grown on permeable filters were used to demonstrate the movement of *Shigella flexneri* from apical to basolateral compartments through paracellular pathways opened by the movement of polymorphonuclear neutrophils from basolateral to apical compartments (Perdomo *et al.*, 1994). Many other examples of the use of this technology to investigate infection in polarized epithelial cells can be found (virus literature to 1993 reviewed in Tucker, Compans, 1993).

Polarized cell culture models have been used to show polarized infection by herpes simplex virus (HSV-1) of MDCK cells (Hayashi, 1995; Tugizov *et al.*, 1996; Topp *et al.*, 1997) but not retinal pigment epithelial (RPE) cells (Tugizov *et al.*, 1996; Topp *et al.*, 1997). Similarly, culture of RPE (Tugizov *et al.*, 1996) and Caco-2 cells (Jarvis *et al.*, 1999; Esclatine *et al.*, 2000) on culture inserts have been used to determine susceptibility of apical or basolateral membranes to HCMV infection and directional release of progeny virus (Tugizov *et al.*, 1996; Jarvis *et al.*, 1999; Esclatine *et al.*, 2000).

Using the culture techniques developed in the previous chapter, I used tight-junctioned confluent trophoblasts grown on semi-permeable artificial membranes to investigate directional release of HCMV progeny virus. I show that the majority of

infectious progeny virus remains predominantly cell-associated as previously found in solid substratum cultures, with little to no virus detected in the basal compartment.

8.2 RESULTS

8.2.1 Basal release of progeny virus from infected triple-layer trophoblast cultures

To determine if progeny virus from infected trophoblasts could be released in a basal direction, trophoblasts were plated according to the technique developed in Chapter 7.0. Three trophoblast seedings onto 3.0 μm filter membranes were interspersed with three to five day incubations in EGF and cultures were used for experiments when high TER and low ^{14}C-dextran transepithelial diffusion were evident (see Figure 8.1 for a diagram of the culture model). A representative experiment of a total of six performed is shown in Table 8.1 and Figure 8.2. In all six experiments performed, the infection level at the time of evaluation for basal release of progeny virus as determined by IE antigen expression was less than 1% and the number of pp65-positive foci containing 5-10 nuclei per focus was less than 100 (e.g. Table 8.1). Experiments were evaluated only during the period in which TER remained high and ^{14}C-dextran transepithelial diffusion remained low compared to cell-free inserts (e.g. Table 8.1). Most progeny virus was found in the cell lysates with approximately 20% released into the supernatant. In this experiment no virus was detected in the basal compartment (Figure 8.2). This pattern of progeny virus distribution was similar in all experiments with less than 0.1% detected at any time in the basal compartment.

8.2.2 Infected trophoblasts were found directly adjacent to the insert membrane

Trophoblasts cultured on insert membranes using the above culture technique consisted of multiple layers of syncytium with an average thickness of 2.7 cells (see Chapter 7.0). This fact and the very low infection levels detected suggested that the reason for little or no progeny virus detection in the basal compartment was that the infection never reached the trophoblasts in direct contact with the filter membrane. One approach to investigate this possibility was to use the confocal microscope to laterally slice through an insert culture of infected cells to determine if fluorescently-labeled IE-positive or pp65-positive nuclei could be detected in direct contact with the filter membrane. Figure 8.3 clearly shows that pp65-positive nuclei can be detected in the first 0.5 μm above the filter membrane. Approximately 26.5 ± 15.0 % of pp65-positive nuclei were located at the membrane surface. This suggests that the infection does reach the

cells attached directly to the filter. If progeny virus is released basally and is able to bypass the TBM, progression through the pores to the basal chamber is possible.

8.2.3 Viral challenge after the first trophoblast seeding

The second approach to address the question of infection in the cells directly adjacent to the filter was to alter the step at which viral challenge occurred. If infection of the first trophoblast seeding which is clearly not confluent (Figure 7.3A), is followed by two subsequent seedings to create the tight-junctioned electrically resistant syncytial layer, there is a high probability of positive infection in the cell layer next to the filter. The time course of a representative experiment using this protocol of a total of four performed is depicted in Figure 8.4. The initial TER observed two days after viral challenge and one day after the second seeding of trophoblasts demonstrated the lack of tight-junctioned confluence in these cultures (Figure 8.4B). One day after the final seeding of trophoblasts, the electrical resistance was high demonstrating confluence and remained high to the end of the experiment. A significantly higher initial infection level was detected in these cultures compared to cultures infected after the third seeding of trophoblasts (compare Table 8.1 with Figure 8.4A). The ten-fold increase in infection level presumably in trophoblasts located directly adjacent to the filter did not alter the progeny virus distribution pattern first observed in trophoblast cultures infected after the third seeding (compare Figures 8.2 and 8.4C). Most remained cell-associated with less than 0.25% found in the basal chamber. A summary graph depicting the results of all four experiments performed using this protocol is presented in Figure 8.5; the median percentage of virus in each compartment was 13.7% in the apical supernatant, 83.7% in the cell lysates and 0.07% in the basal compartment. The endpoint day for each experiment differed based on the characteristics of each trophoblast preparation and was chosen as the last day after viral challenge still demonstrating high TER and low ^{14}C-dextran transepithelial flux. Increasing the MOI from 10 to 20 did not increase the progeny virus found in the basal compartment (Figure 8.5).

8.3 SUMMARY OF RESULTS

8.3.1 Infection of triple-seeded trophoblast cultures grown on 3.0 μm insert membranes resulted in low infection efficiencies with almost no detectible progeny virus in the basal compartment.

8.3.2 Infected nuclei expressing pp65 antigen were found directly adjacent to the insert membrane surface, thus progeny virus could potentially be released directly through membrane pores.

8.3.3 Infection of the first seeding of trophoblasts followed by seeding two more trophoblast layers resulted in much higher infection efficiencies but again with little detectible progeny virus in the basal compartment. The majority of virus remained cell-associated.

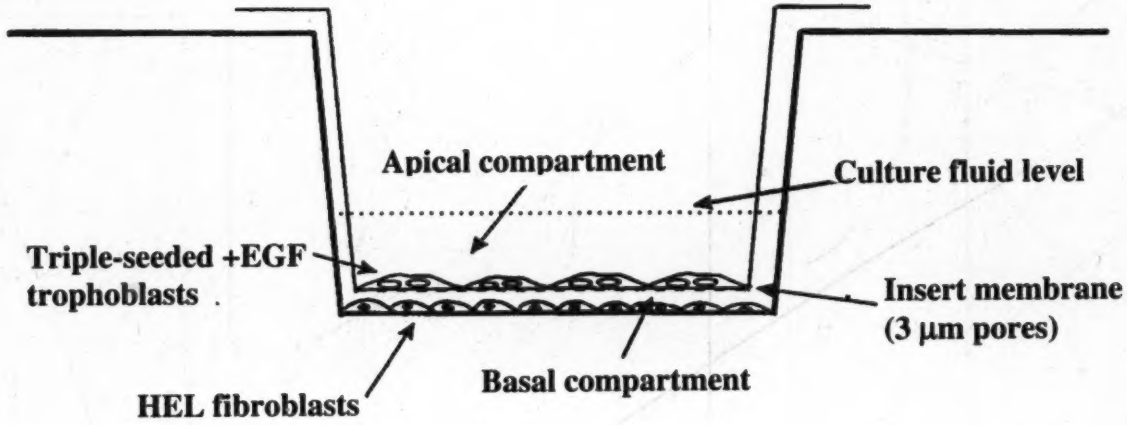


Figure 8.1 Insert culture model

Table 8.1[a] Characteristics of HCMV infected triple-layer trophoblast cultures

IE-positive nuclei (%)[b]	pp65-positive nuclei (foci)[c]	Electrical Resistance (ohms x cm^2)[d]	^{14}C-Dextran Transepithelial Flux (pmoles/hr/cm^2)[e]	
			Cells	Blank
0.95 ± 0.05	44.5 ± 2.12	54.2 ± 12.4	0.18 ± 0.01	9.12 ± 0.70

a Results at 11 days after viral challenge are presented as the average ± SD and are representative of six independent experiments.

b Culture inserts were prepared with three layers of trophoblasts as described in Materials and Methods, infected with AD169 at an MOI of 10 and the percent IE-positive nuclei in two replicate inserts determined.

c The number of pp65-positive foci in two replicate inserts.

d TER was measured in five replicate inserts and the value calculated by subtracting the average value for cell-free inserts (22.8 ohms x cm^2).

e Transepithelial flux of ^{14}C-dextran was measured in the first 15 minutes through inserts containing infected triple-layer trophoblasts (cells) or cell-free inserts (blank).

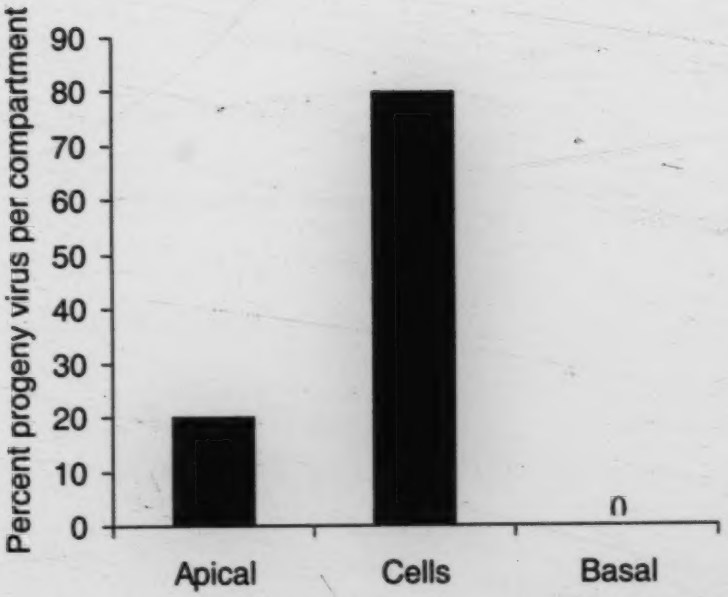


Figure 8.2 Progeny virus remains predominantly cell-associated in infected trophoblast insert cultures.
The culture inserts described in Table 8.1 were assessed for progeny virus as follows: at 10 days after viral challenge, the inserts were washed well, refed with culture medium and placed into wells containing confluent HEL fibroblasts. 24 hours later insert supernatants were removed (apical compartment; 9 inserts) and the infected trophoblast cultures treated with a low pH saline solution to remove adherent virus and cell lysates prepared (cells compartment; 2 inserts). The supernatants and cell lysates were assayed for progeny virus as described in the Materials and Methods. The wells containing HEL cells were cultured an additional 18 hours and immunohistochemically stained for IE antigen to detect any virus reaching the basal compartment (9 inserts). The average amount of virus in each compartment was calculated. The distribution of virus between compartments is depicted as a percentage of total virus derived from the three averages.

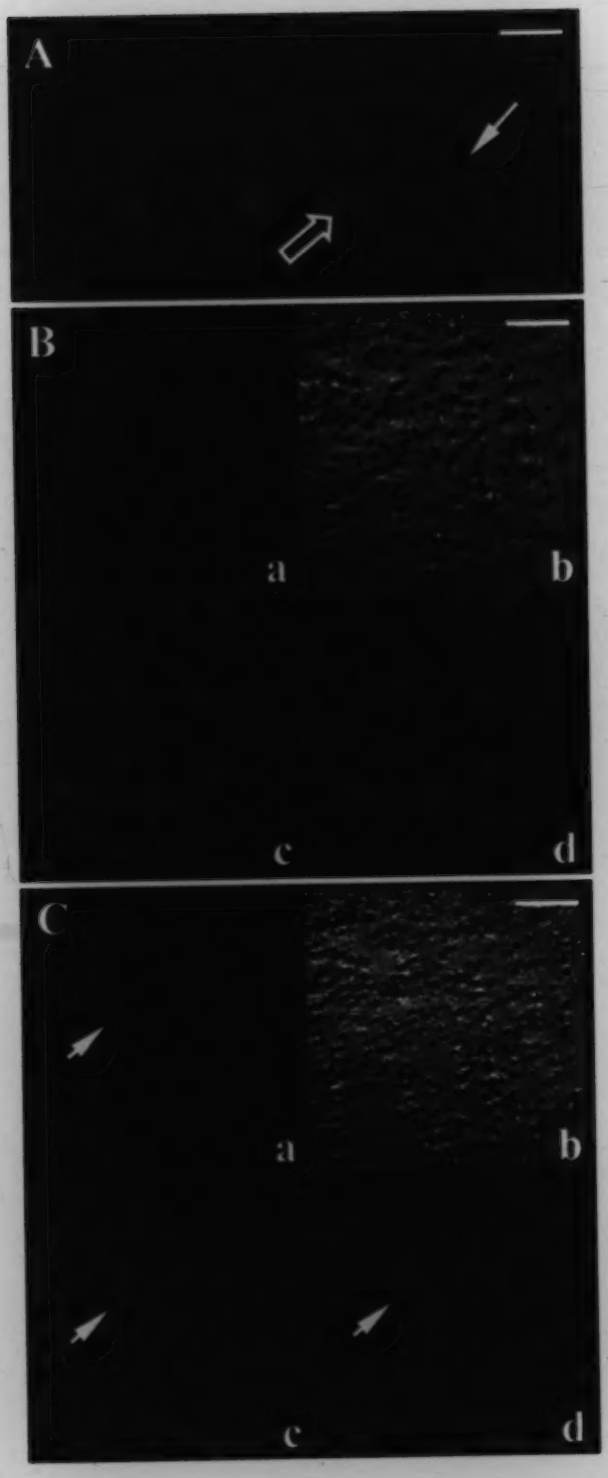

A
B
a
b
c
d
C
a
b
c
d

Figure 8.3 Infection reaches trophoblasts directly adjacent to the insert membrane. Infected triple-layer trophoblast insert cultures were prepared as described in Table 8.1. At 11 days after viral challenge, cultures were stained with a primary antibody to HCMV pp65 antigen which was detected using a secondary fluorescent-labelled probe (Alexa Fluor 546). Nuclei were counterstained with DAPI and results visualized using a Zeiss confocal fluorescent microscope. **(A)** Insert was scanned at the level of the insert surface (green) showing pores in focus (solid white arrow) and pp65-positive nuclei (open white arrow). Bar = 12 μm. **(B)** and **(C)** are scans of the same field taken at different depths relative to the insert surface; 11.1 μm above **(B)** and 0.5 μm above **(C)**. Bar = 30 μm. Short solid white arrows denote a pp65-positive nucleus in (a) Alexa Fluor 546 scan (c) DAPI scan and (d) combined Alexa Fluor 546 and DAPI scan. (b) the image by light microscope showing the insert membrane in focus in **(C)** but not in **(B)**.

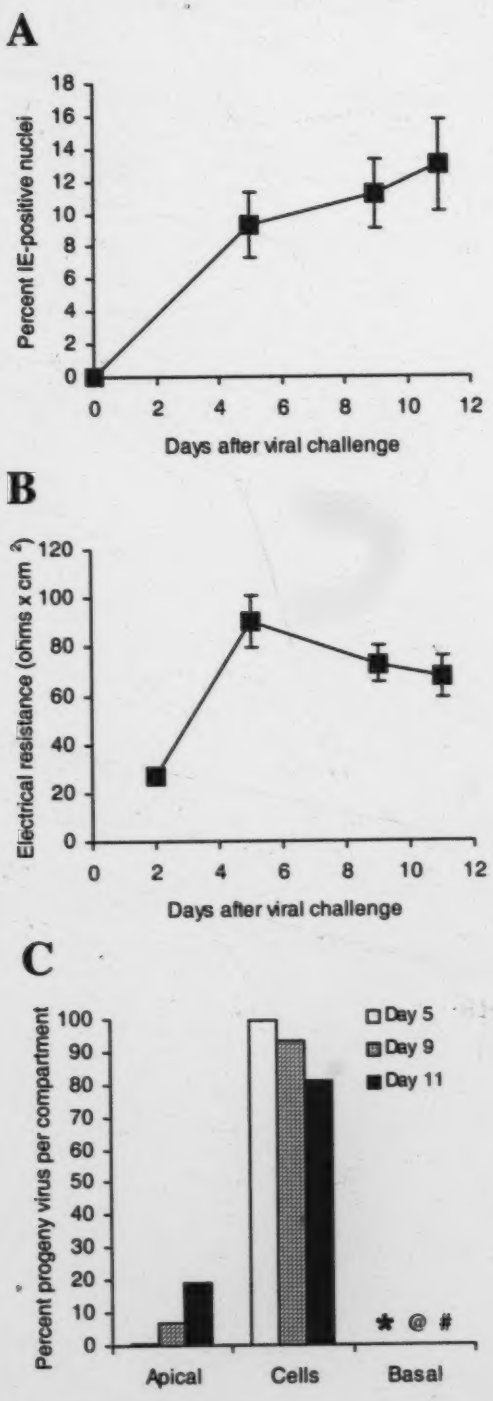

A
Percent IE-positive nuclei
18
16
14
12
10
8
6
4
2
0
0 2 4 6 8 10 12
Days after viral challenge
B
Electrical resistance (ohms x cm 2)
120
100
80
60
40
20
0
0 2 4 6 8 10 12
Days after viral challenge
C
Percent progeny virus per compartment
100
90
80
70
60
50
40
30
20
10
0
Day 5
Day 9
Day 11
* @ #
Apical
Cells
Basal

Figure 8.4 Time course of infection in culture inserts infected after the first trophoblast seeding.

Trophoblasts were cultured on inserts for three days in the presence of EGF and challenged with AD169 at an MOI of 10 for 24 hours. A low pH treatment to remove adherent virus was performed prior to the application of a second layer of trophoblasts. A third layer of trophoblasts followed three days later. Cultures were assessed on days 5, 9 and 11 after viral challenge as follows: **(A)** Average ± SD of percent IE-positive nuclei was determined in three replicate inserts; **(B)** TER was measured in seven replicate inserts and the value calculated by subtracting the average value for cell-free inserts (22.8 ohms x cm^2); Where error bars are not seen, error was less than the width of the marker or zero. **(C)** Distribution of progeny virus in each compartment as described in the legend to Figure 8.2 was determined. Values for the basal compartment are: ✱ = 0.04%; @ = 0.07%; # = 0.21%. Results are representative of four independent experiments.

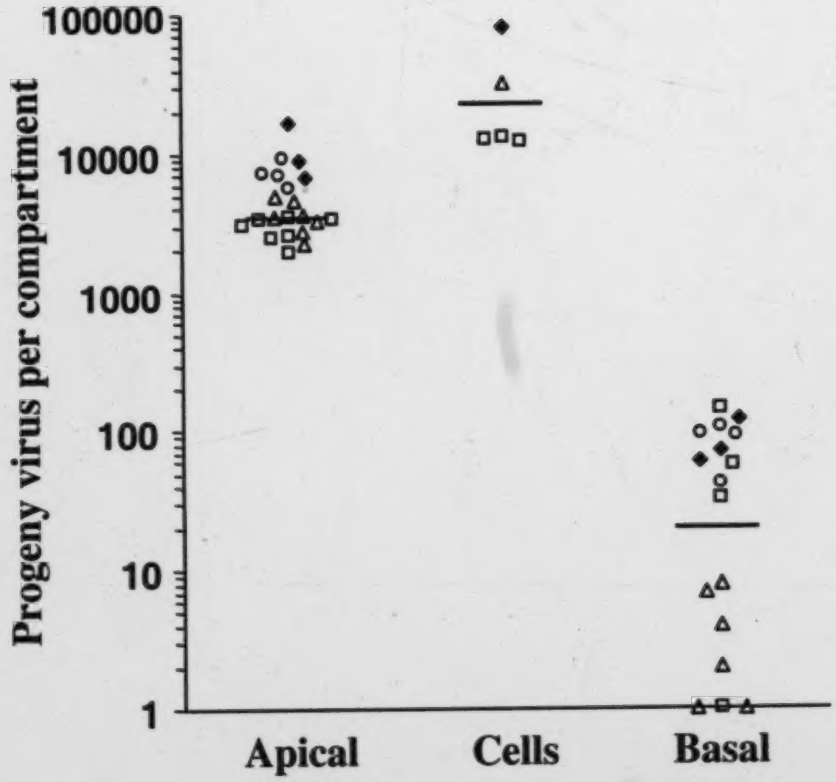


Figure 8.5 Summary of the distribution of progeny virus in apical, cellular and basal compartments of trophoblast insert cultures infected after the first seeding of cells.

Compilation of results from four independent experiments of trophoblasts infected on inserts as described in the legend to Figure 8.4 are depicted. Progeny virus in each compartment was determined as described in the legend to Figure 8.2. The infection levels as determined by percent IE-positive nuclei ranged from 13.0 to 25.0%. The day of assessment after viral challenge varied with each experiment and was dependent on continuance of high TER and low ^{14}C-dextran transepithelial diffusion: □ challenged at an MOI of 20, assessed on Day 11; △ challenged at an MOI of 20, assessed on Day 8; ◆ challenged at an MOI of 10, assessed on Day 18; ○ challenged at an MOI of 10, assessed on Day 8. Median values are: Apical = 3522, Cells = 22250 and Basal = 20.

CHAPTER 9.0 DISCUSSION

9.1 INTRODUCTION

During pregnancy the ST that envelopes the chorionic villi and directly contacts maternal blood within the intervillous space provides the fetus with more than just nutrients and gas exchange. Along with its TBM, it also acts as a physical barrier to potentially harmful maternal immune cells and provides a measure of protection from maternal blood-borne pathogens (reviewed in Benirschke, Kaufmann, 2000c). One of the potential mechanisms by which a pathogen could bypass an intact ST barrier to gain access to the fetus would be by direct infection. Three possible outcomes of a direct infection of this cell layer are: (1) the pathogen is restricted within the barrier because of inefficient replication and/or induction of latency; (2) the pathogen replicates but is contained within the ST because egress is blocked; or (3) the pathogen is effectively reproduced and released in a basal direction towards the fetus either by egress from the intact barrier or as a result of focal damage induced by the infection.

HCMV is a blood-borne pathogen found cell-free during bouts of viremia but otherwise generally found cell-associated in polymorphonuclear cells and monocytes (reviewed in Mocarski, 1996). This virus is transmitted to the fetus resulting in a frequently asymptomatic congenital infection but symptoms can range from mild neurological damage and hearing loss to the severe manifestations observed in cytomegalic inclusion disease (CID) including death (reviewed in Stagno, 1995). The mechanism of transmission of HCMV is unknown but is thought to involve infection of the placenta since villitis is often observed in pregnancies complicated by transmission of this virus (reviewed in Blanc, 1961; Garcia *et al.*, 1989). Is fetal infection initiated by infection of the villous ST which leads to infection of cells within the chorionic villi? This is the overall question addressed in this thesis. The results from all five data chapters are discussed in a topical manner followed by a general discussion and presentation of a model describing one possible mechanism for vertical transmission of HCMV.

9.2 PERMISSIVE HCMV INFECTION OF PRIMARY VILLOUS TERM AND FIRST TRIMESTER TROPHOBLASTS

The results of my initial studies to determine if cultured villous trophoblasts could be permissively infected with HCMV are discussed here. A survey of the literature investigating placental involvement associated with symptomatic congenital HCMV infections in third trimester placentas showed that while permissive infection could be detected in chorionic villous stromal cells, only rarely was it found in trophoblasts (Table 1.1; Quan, Strauss, 1962; Hayes, Gibas, 1971; Monif, Dische, 1972; Benirschke *et al.*, 1974; Mostoufi-Zadeh *et al.*, 1984; Garcia *et al.*, 1989; Muhlemann *et al.*, 1992; Sinzger *et al.*, 1993; Nakamura *et al.*, 1994; Schneeberger *et al.*, 1994). *In vitro* a permissive infection in trophoblasts was only shown to occur upon co-infection with another virus, either HIV-1 (Toth *et al.*, 1995b), HTLV-1 (Toth *et al.*, 1995a) or by coculture with macrophages (Bacsi *et al.*, 1999). In this thesis I demonstrate that cultured trophoblasts can be readily and permissively infected with HCMV but require a high HCMV inoculum, infection progresses slowly compared to fibroblasts and progeny virus remain predominantly cell-associated.

The cell isolation procedures and culture models used in this study were essential for a complete characterization of trophoblast infection by HCMV. The ability to maintain viable cultures for greater than three weeks without overgrowth by proliferating placental fibroblasts was crucial for demonstration of the productive but slowly progressing infection in trophoblasts (Figure 4.3A). Direct demonstration of IE and pp65 antigen positive cells that were vimentin negative (thus trophoblasts; Figure 4.3B) eliminated the possibility that the low HCMV infection frequencies initially observed were due to preferential infection of placental fibroblasts.

Interestingly, most of the very few vimentin-positive cells (fibroblasts) in these cultures were uninfected. Possible reasons include: (a) a disadvantageous target ratio (there are >4000-fold more trophoblasts), (b) infected vimentin-positive cells may lyse and not be detected, although lack of high titers of infectious virus in supernatants during the first week of culture argues against this or (c) fibroblasts, which strongly adhere to tissue culture plastic, may lie beneath the trophoblasts and be protected from virus challenge.

HCMV crosses the placenta at all stages of gestation (Griffiths, Baboonian, 1984; Stagno *et al.*, 1986; Yow *et al.*, 1988; Demmler, 1991) and villous trophoblasts from first trimester placentas show signs of permissive infection *in vivo* (Garcia *et al.*, 1989; van Lijnschoten *et al.*, 1994) and more recently *in vitro* (Amirhessami-Aghili *et al.*, 1989; Fisher *et al.*, 2000; Gabrielli *et al.*, 2001). Rosenthal et al (Rosenthal *et al.*, 1981), however, found that HCMV infected first trimester placental fibroblasts but not trophoblasts. I confirmed that first trimester placental fibroblasts were readily infected (Figure 4.10) but also found that +EGF first trimester trophoblasts were infected (Figure 4.8). The infection of first trimester trophoblasts was intermediate between placental fibroblasts and +EGF term trophoblasts in two aspects: the fraction of cells infected at near saturating virus titers and the kinetics of the infection. Twenty-four hours after a two-hour viral challenge all placental fibroblasts were IE-antigen positive at an MOI of 3.5, 15% of +EGF first trimester trophoblasts at an MOI of 16 and only 6% of +EGF term trophoblasts at an MOI of 38 (Figure 4.9). The kinetics of progression from the immediate-early to early-late infection stage could be visualized by plotting the ratio of pp65 to IE-positive foci in cultures as a function of time (Figure 4.10). After HCMV challenge of placental fibroblasts at an MOI of 0.19, measurable pp65 antigen was observed within 24 hours and over half of infected cells had progressed to the early-late stage by 48 hours. In contrast, even at a challenge MOI of 1, pp65 antigen did not appear in +EGF term trophoblasts until after day 4 and the ratio of pp65/IE never exceeded 0.3 over a 21-day culture period. +EGF first trimester trophoblasts showed intermediate progression kinetics. At an MOI challenge of 1, pp65 antigen expression appeared within 24 hrs but thereafter progression was slower than in fibroblasts with a ratio of pp65/IE approximately 0.4 nine days after challenge. Although the viral tegument protein pp65 enters the nucleus immediately upon infection of a cell (Yamauchi *et al.*, 1985), the antibody used in these assays could not detect pp65-positive nuclei from virus inoculum prior to 24 hours, suggesting that the detected pp65 was produced *de novo* (Figure 4.2A). The delay in progression to a late stage of infection in HCMV infected term trophoblast cultures has been confirmed (Halwachs-Baumann *et al.*, 1998). The larger fraction of infectable cells and more rapid progression kinetics may explain why more first trimester

than term villous trophoblasts with permissive infection are detected *in vivo* (Garcia *et al.*, 1989; van Lijnschoten *et al.*, 1994).

I also find that unlike fibroblasts where infectious progeny virus was readily released (Table 6.3; Mocarski, 1996), virus remained predominantly cell-associated in both term and first trimester trophoblasts (Figures 4.4, 4.8, 6.2, 8.4, 8.5 and Table 6.1). HCMV was also found to be exclusively cell-associated in infected monocyte-derived macrophages, specifically in cytoplasmic vacuoles (Fish *et al.*, 1995), in brain-derived cultures (Jault *et al.*, 1994) and in a rhabdomyosarcoma cell line (Cinatl *et al.*, 1994). A placental barrier that retains infectious progeny virus is in accord with studies in the guinea pig model (Griffith *et al.*, 1985; Goff *et al.*, 1987) showing that the placenta can accumulate CMV without transmission to the fetus.

My results differ from those of Toth et al who found that HCMV infection of syncytialized term trophoblasts was abortive and became fully permissive only if the cells were pre-infected with HIV-1 (Toth *et al.*, 1995b), co-infected with HTLV-1 (Toth *et al.*, 1995a) or cocultured with placental macrophages (Bacsi *et al.*, 1999). The reasons for the disparate results are not clear but it is possible that different CT subpopulations were isolated by the slightly different negative selection methods used in the two laboratories: elimination of MHC class I, MHC class II and CD9 expressing cells in my laboratory (Kilani *et al.*, 1997) and elimination of MHC class I and II cells in their laboratory (Toth *et al.*, 1995b). In all three papers the authors were able to detect HCMV IE and early antigens in term trophoblast cultures infected with HCMV alone, but no late antigens although no time course for the latter was shown. Given these results and the inability to detect progeny virus in cell culture supernatants, they concluded there was no permissive infection (Toth *et al.*, 1995a; Toth *et al.*, 1995b; Bacsi *et al.*, 1999). However, based on my results showing predominantly cell-associated progeny virus in trophoblasts, they may have missed detection of permissive infection by failing to test for progeny virus associated with cell lysates. Additionally, it was unclear whether expression of HCMV late antigen was evaluated late in infection, which would be essential according to my results. As well, another group looking at dual infection with HIV-1 and HCMV in brain cell cultures found, unlike Toth et al, that HIV-1 did not affect HCMV replication in permissive or nonpermissive cell lines and if both viruses were permissive, dual infection

would result in repression of HIV-1 (Jault *et al.*, 1994). An alternative explanation for the differences observed is that the laboratory strain of HCMV, AD169, used in both studies, could be substantially different since, according to Cha et al (Cha *et al.*, 1996), long-term passage can result in loss of genetic information, explaining differences in tissue tropism and virulence. To confirm that the permissive infection I observed was not a property of my laboratory AD169 strain, I tested two other well-known laboratory-adapted strains, Towne and Davis and two low passage congenital isolates. Although there was considerable variation in infection efficiency (AD169, Towne and Kp7 infected much more efficiently), all strains were able to permissively infect trophoblasts (Tables 4.1 and 6.3).

The ST is a rather unique tissue in that it is a continuous, multinucleated cell layer that, theoretically, covers entire villous branches. The +EGF cultures in this study, although not continuously syncytialized, nonetheless offered a useful model of the ST. An average of 64% of nuclei are found in multinucleated cells with >2 nuclei and approximately 20% are in cells with as many as 50 nuclei (Kilani *et al.*, 1997). Although the results depicted in Table 5.1 demonstrate a lower level of multinucleation, there is considerable variation between placental preparations with some taking longer than five days to achieve the above average. It was consistently observed that either all, or no, nuclei in HCMV-challenged syncytialized cells were positive for HCMV antigens (Figure 4.1A). Since syncytium, both in culture and *in vivo*, does not proliferate (Aplin, 1991; Garcia-Lloret *et al.*, 1996), any increases in the number of infected nuclei must have come from free virus infection, fusion of infected with uninfected cells or cell to cell transmission (focal spread; Pulliam, 1991; Navarro *et al.*, 1993). I found that the spread of virus was initially focal since the number of infected nuclei increased but the number of foci did not (Figures 4.2B, 4.5). An increase in the number of foci coincided with release of progeny virus into culture supernatants, suggesting that free virus dissemination could also occur. Tugizov et al have shown that lateral spread of HCMV infection in RPE cells may involve different viral glycoproteins, suggesting that lateral spread and apical infection are distinctly different (Tugizov *et al.*, 1996). In some cell types, lateral spread does not occur but preferential basolateral infection does (Jarvis *et al.*, 1999). In the absence of trophoblast proliferation in culture, cell loss due to death or

shedding leads to a decrease in DNA content in uninfected cultures over time (Yui *et al.*, 1994). Infection of +EGF and –EGF cultures did not increase this loss of DNA content, suggesting that infected cells are not preferentially lost when infection frequencies are low (Figure 4.7).

9.3 INFECTION OPTIMIZATION AND RESIDUAL INOCULUM

The initial studies of HCMV infection in cultured trophoblasts resulted in permissive but inefficient infection of primary villous trophoblast cultures using a standard two-hour viral challenge time (Chapter 4.0). Increasing the low incidence of infection in trophoblast cultures was essential for further investigations into the effects of infection on the barrier function of differentiated trophoblasts. Increasing the viral challenge time to 24 hours in the presence of 2% FBS resulted in optimum infection levels in both +EGF and –EGF trophoblast cultures (Figures 5.1, 5.2A). An increase in infection efficiency was also detected in fibroblast cultures (Figure 5.2A) by increasing the time of viral challenge as has been previously shown using murine CMV (Hodgkin *et al.*, 1988). A modest increase in trophoblast infection was also observed by increasing the MOI from 2.2 to 10 (see the 24-hr challenge times in +EGF cultures: 3.1% compared to 9.9% in Figures 5.1 and 5.2A respectively). This confirmed the slight increase in infection levels observed in +EGF term trophoblast cultures when tested over a range of MOIs using a two-hour viral challenge time (Figure 4.9). HCMV plaque number has been shown to increase, not by increasing the total amount of virus, but by increasing virus concentration (Hodgkin *et al.*, 1988). All MOIs in this thesis were diluted in exactly the same volume so any increase in MOI resulted in increased virus concentration and not just increased PFU.

Surprisingly, viral challenge for 24 hours at an MOI of 10 resulted in adherence of viral inoculum that was not immediately evident, i.e. a final wash directly after the 24-hour challenge showed little infectious virus but within 24 to 48 hours the culture supernatant contained significant amounts (Table 6.1). This released infectious virus was unlikely to be due to progeny virus since in trophoblasts (Figure 4.4) and even in fibroblasts (Mocarski, 1996), release of progeny virus is delayed for at least 72 hours. In a placental perfusion model of infection, Muhlemann et al also suggested that high levels

of virus detected in the perfused placental tissues after 9.5 hours with no accompanying evidence of infection may have been due to adherence of inoculum (Muhlemann *et al.*, 1995). I do note, however, that the recirculating system contained heparin, which is able to compete with HCMV for binding to heparan sulfate moieties and may also inhibit infection (Compton *et al.*, 1993). Adherent virus being slowly released after basolateral viral challenge but prior to progeny virus production was also seen in single-step virus growth curves of infected Caco-2 cells (Jarvis *et al.*, 1999).

HCMV is heat-labile with a half-life of 60 minutes at 37°C in cell-free medium but persists in co-culture with trophoblasts (Figure 5.2B, Table 5.1; Vonka, Benyeshmelnick, 1966). An increase in the number of cells expressing IE antigen after a 24-hour viral challenge and the adherence of inoculum even after thorough washing, suggested that the virus was quickly protected from inactivation by an initially stable attachment to cell surface components such as heparan sulfate proteoglycans (HSPGs; Compton *et al.*, 1993; Compton, 1995). *In vivo*, ST but not CT express syndecan-1 (Jokimaa *et al.*, 1998), a member of one family of HSPGs, which could explain the enhanced adherence of viral inoculum to +EGF trophoblasts compared to –EGF trophoblasts. A more stable attachment to a higher affinity receptor such as CD13 or annexin II follows the initial binding to HSPGs, which then leads to entry by pH-independent fusion (Compton *et al.*, 1992; Compton, 1995). A delay in this second binding step in conjunction with spontaneous dissociation and reassociation to HSPGs could explain both the improved infection efficiency by increasing viral contact time and the initial adherence of inoculum followed by later detection in culture supernatants.

Viral binding of mouse CMV to fibroblasts has been shown to occur at three levels: loosely adherent virions could be removed simply by washing the cells; more tightly bound virions remained adherent after the wash but were released into the supernatant gradually over a five-minute time period and a third group of virions were irreversibly bound (Hodgkin *et al.*, 1988). The longer virus was in contact with the cells, the more irreversibly bound virus was detected. This short five-minute dissociation step in fibroblasts is likely why I do not detect residual virus after 24 hours in infected fibroblast cultures (Table 6.2); all virus has reassociated and become irreversibly bound in that time period to these highly susceptible cells. In trophoblast cultures dissociation

and reassociation or alternatively irreversible binding may be slower than in fibroblasts resulting in detectable residual virus 24 hours after washing and lower infection efficiencies. Centrifugation further increased the infection efficiency in trophoblasts and fibroblasts presumably because association rates were increased. The longer virus remains in contact with the cell, the higher the probability of binding irreversibly to the higher affinity receptors as suggested in the murine system (Hodgkin *et al.*, 1988). Protection of the virus by reversible binding along with a delay in the irreversible binding step suggests that the placenta could indeed be a viral reservoir (Griffith *et al.*, 1985). Further studies are needed to confirm and expand these initial results.

Changing the stock virus preparation method may have improved the PFU to non-infectious particle ratio since repeated freezing and thawing (Benyesh-Melnick *et al.*, 1966) inactivates infectious HCMV. Since non-infectious particles and dense bodies could compete with infectious virus for binding to cells (Fortunato *et al.*, 2000) the greater the number of infectious virions the greater the chance of successful penetration and cellular infection. Non-infectious particles can also trigger activation of IFN-inducible genes that could potentially interfere with a productive infection by infectious virus. Thus an improved PFU to non-infectious particle ratio could contribute to the improved infection levels observed after Chapter 4.0.

HCMV like other herpesviruses is sensitive to low pH (Kempf *et al.*, 1991) and treatment of virally challenged cells with a low pH solution for a short duration inactivated adherent virus without cell loss (Table 6.1, Figure 6.1; Compton, 2000). Incubation of the cultures for two days after viral challenge with IVIG, which contains neutralizing antibodies to HCMV was also able to remove residual virus, likely by binding to the virus as it dissociated and preventing its reassociation. Even though residual inoculum did not appear to affect the final outcome of experiments done in solid substratum cultures, removal of it using a low pH wash following the 24-hour viral challenge and at all times just prior to cell lysate preparation was done to allow accurate interpretations of basal release studies and for accurate estimates of cell-associated progeny virus.

9.4 SUSCEPTIBILITY OF PRIMARY VILLOUS TROPHOBLASTS TO HCMV INFECTION

The susceptibility of +EGF and –EGF trophoblast cultures to HCMV infection in initial experiments using an inefficient viral challenge protocol was low and did not differ (Figures 4.5 and 4.6). Upon optimization of viral challenge, not only did initial infection frequencies in both trophoblast cultures increase, but a significant difference in susceptibility was also noted (Figure 5.3). As described in the results, these differences may in fact be more pronounced if the number of infected cells is compared rather than the number of infected nuclei. The majority of trophoblasts in either culture, however, remained resistant to HCMV infection. Although IE-antigen expression is not necessarily indicative of a permissive infection (Sinzger *et al.*, 1996), it provides a means of identification and comparison of cells able to initiate the infection from those that can not. Infection progression to later stages in these cultures is discussed in Section 9.5.

Unlike most cell types investigated *in vitro* (Tanaka *et al.*, 1984; Gonczol *et al.*, 1985; Smith, 1986; Weinshenker *et al.*, 1988; Poland *et al.*, 1990; Lathey, Spector, 1991; Ibanez *et al.*, 1991; Poland *et al.*, 1994; Tugizov *et al.*, 1996) and in some *in vivo* studies (Pulliam, 1991; Sinzger *et al.*, 1996) where a more differentiated phenotype was essential to susceptibility and permissive infection, the less differentiated trophoblast cultures appeared to be more susceptible to HCMV. The number of nuclei expressing IE antigen in infected –EGF trophoblast cultures was significantly greater than in infected +EGF cultures (Figure 5.3) even when viral challenge was done on later days of culture (Figure 5.5). Since EGF serves both to accelerate a differentiation process that is ongoing in –EGF cultures and to induce extensive syncytialization (Morrish *et al.*, 1987; Yui *et al.*, 1994; Kilani *et al.*, 1997), as both cultures age one would expect the +EGF cultures to mature more rapidly than the –EGF cultures. If susceptibility to HCMV infection is inversely related to trophoblast maturation (Figure 5.3), the decreasing infectibility observed that was more pronounced in the +EGF cultures is not unexpected (Figure 5.5).

Cells that are active and proliferating tend to be more readily infected by HCMV (Stinski, 1977) and treatments that decrease cell metabolism such as actinomycin D (Landini *et al.*, 1979) or ultraviolet irradiation (Furukawa *et al.*, 1975) inhibit infection. Although primary isolated trophoblasts do not proliferate in culture (Contractor,

Sooranna, 1982; Kliman *et al.*, 1986; Aplin, 1991; Yui *et al.*, 1994; Garcia-Lloret *et al.*, 1996), they are metabolically active (Yui *et al.*, 1994). To confirm that a decrease in metabolic activity as cultures aged did not account for the decreased infectivity, metabolic capacity was measured in aging uninfected cultures. Both +EGF and –EGF trophoblast cultures remained metabolically active over long-term culture periods (Figure 5.6).

The differences observed in infection levels between +EGF and –EGF cultures are likely much greater than depicted if the numbers are reevaluated based on cells rather than nuclei. For example, in Table 5.1 34.7% of cells in +EGF cultures were multinucleated with an estimated average of 4 nuclei per cell (Kilani *et al.*, 1997). Viral challenge MOIs were based on the total number of nuclei in each trophoblast culture not cells. An actual MOI for +EGF cultures based on 4 nuclei per cell in 35% of the culture would be approximately 14 compared to 10 in –EGF cultures, emphasizing that the differences in infection frequencies were underestimated.

To ensure that the presence of EGF during viral challenge was not responsible for infection inhibition in +EGF cultures, I challenged –EGF trophoblast, +EGF trophoblast and fibroblast cultures in the presence or absence of EGF. There was clearly no negative effect on HCMV susceptibility if EGF was included (Figure 5.4). In fact enhanced infections were observed both in -EGF trophoblast and fibroblast cultures challenged in the presence of EGF (Figures 5.4). EGF could be acting either directly or indirectly to increase susceptibility to HCMV infections. EGF through binding to its cellular receptor could act directly by affecting viral association/dissociation rates, higher affinity receptor binding, fusion and entry or IE antigen expression. For example, EGF through activation of its receptor stimulates NF-κB activation (Hoffmann *et al.*, 1998; Biswas *et al.*, 2000; Zelenaia *et al.*, 2000) and the HCMV IE promoter contains a number of NF-κB binding sites (Sambucetti *et al.*, 1989) suggesting one mechanism by which EGF could enhance trophoblast infection. The lack of enhancement observed in cultures pretreated with EGF (+EGF cultures) may reflect the lingering effects of EGF on trophoblast maturation even though the growth factor itself had been washed away.

Alternatively, the effect of EGF on infection in cultures of immature trophoblasts might in fact be indirectly due to the cellular maturation induced by EGF and not due to

EGF itself. Although trophoblasts pretreated with EGF for five days are operationally considered to be differentiated, even short-term treatment with EGF results in changes consistent with a more differentiated phenotype (Morrish *et al.*, 1987; Morrish *et al.*, 1997). This fact and the increased susceptibility in trophoblasts cultured for only one or three days with EGF compared to –EGF trophoblasts (Figures 5.4 and 5.5A) suggested that maturation could be important. Thus a particular maturation state may be essential for HCMV susceptibility and this state is more prevalent in –EGF cultures and young +EGF cultures than in older +EGF cultures.

When trophoblast cultures were examined by immunohistochemistry for cell-specific distribution of differentiation markers such as syncytialization or PLAP expression, it was clear that variable states of differentiation existed within each culture even though –EGF cultures were predominantly immature and +EGF cultures predominantly more differentiated overall (Figure 5.7A versus 5.7E, Table 5.1; Yui *et al.*, 1994). The inverse relationship of percent PLAP-positive cells to percent IE-positive cells in either trophoblast culture (Figure 5.8) and the predominance of infection in PLAP-negative or intermediately stained trophoblasts rather than intensely stained areas (Figure 5.9) suggested that an immature or early differentiation state was more susceptible than terminally differentiated cells.

The observation that multinucleated cells appeared to be more readily infected than mononucleated cells particularly in –EGF cultures appears to contradict this conclusion if we accept that multinucleation is a hallmark of advanced differentiation in these cultures (Table 5.1). An alternative explanation is that the particular maturation state that is most susceptible to HCMV infection may include mononuclear cells that are on the verge of fusing and recently fused cells. Thus –EGF cultures and young +EGF cultures contain more susceptible cells and are consequently more vulnerable to infection than older +EGF cultures that contain more terminally differentiated cells.

Both the PLAP and syncytialization data suggest that the maturation state of an individual cell and not the particular culture condition in which it is found is important to HCMV susceptibility. The number of cells in this particular state of maturation likely varies between trophoblast preparations, as does PLAP expression (Figure 5.7), resulting in differential HCMV susceptibility between preparations. Further examination of this

fascinating possibility will lead to a greater understanding of the mechanism by which this virus is able to reach the fetus.

Several studies suggest that a differentiated phenotype is not always required for HCMV susceptibility (Michelson-Fiske *et al.*, 1975; Knowles, 1976; Tumilowicz *et al.*, 1985; Lathey *et al.*, 1990; Poland *et al.*, 1994; Cinatl *et al.*, 1994; Riegler *et al.*, 2000). A study of HCMV infectivity in an intestinal epithelial cell line Caco-2 suggests that like trophoblasts, susceptibility to infection as determined by IE-antigen expression is dependent on early rather than late stages of differentiation (Jarvis *et al.*, 1999). Infection of this cell type occurred preferentially through the basolateral membrane of insert cultures with TER of 250 ohms x cm^2 or less. Loss of susceptibility occurred as the cultures further differentiated to a TER of 350 ohms x cm^2 or more. The authors demonstrated that loss of infectibility was at the level of viral entry and could be rescued by treatment of differentiated cultures with EGTA, which disrupted cell-to-cell contact. They concluded that HCMV receptors were sequestered in highly differentiated cultures and inaccessible to the virus (Jarvis *et al.*, 1999).

It would be interesting to determine if a similar mechanism operates in trophoblast cultures. Although all viral challenges were performed apically, it is possible that the basolateral membranes are more susceptible to infection by HCMV. The trophoblast culture model developed in this thesis to allow access to both specialized epithelial membranes makes it possible to test for preferential basolateral infection. Triple-seeded insert cultures are multilayered with tight junctions and virus applied to the apical surface would be unlikely to reach basolateral surfaces thus providing a true measure of apical infection. One way to expose the basolateral surface in these cultures may be to use EGTA (Pitelka *et al.*, 1983) to disrupt the tight junctions as suggested above (Jarvis *et al.*, 1999). Alternatively, viral challenge could be done in the basal chamber to presumably reach the basolateral surfaces. Interpretation of any results would, however, be complicated by the fact that approximately 15% of the underside of the semi-permeable insert membrane (basal facing) is covered with parts of anucleated trophoblast pseudopods that have proceeded through the 3 μm pores. Whether this 15% represents an apical or basolateral surface, 85% of the semi-permeable insert membrane remains cell-free allowing for basolateral infection of the trophoblasts. I have preliminary

evidence that at least 50% of the pores are free of invading trophoblast pseudopods and that an electron dense layer that likely represents the TBM covers but does not proceed into the pores, suggesting a true basolateral aspect available for infection. Further investigation should be done using the scanning electron microscope to view the entire basal surface of the insert. Exploration by staining or analyzing the activity of proteins or enzymes specific to the apical or basolateral membranes would further define the culture model and permit more extensive investigations.

The level at which HCMV infection is blocked in the majority of trophoblasts is currently unknown. Transduction with recombinant adenovirus vectors was significantly reduced upon trophoblast differentiation. This reduction was dependent on resistance to adenovirus infection and not due to reduced transcription or translation efficiency as determined by transient transfection (MacCalman *et al.*, 1996). In many undifferentiated cell types the inability to replicate HCMV is at the level of major immediate early promotor (MIEP) and not at the level of viral entry or access to the nucleus (Nelson *et al.*, 1990). One way to investigate the block, as described in the Caco-2 paper (Jarvis *et al.*, 1999), is to compare the number of IE-positive cells after normal viral infection to the number of green fluorescent protein (GFP) positive cells after infection with a recombinant HCMV constitutively expressing GFP (Vieira *et al.*, 1998). Expression of GFP is controlled by the constitutively active promoter for the cellular elongation factor 1α (EF-1α). This would determine if differences in HCMV susceptibility were due to lack of entry (i.e. the numbers of GFP-positive and IE-positive cells should be the same) or at some point after entry (the number of GFP-positive cells would be greater than the number of IE-positive cells) (Jarvis *et al.*, 1999). A cellular block on IE promoter (MIEP) activity after viral entry could be assessed by transient transfection and comparison of the transcriptional activity of two promoters such as MIEP and EF-1α using a reporter gene such as GFP.

9.5 PROGRESSION OF HCMV INFECTION IN PRIMARY VILLOUS TROPHOBLAST CULTURES

Permissive HCMV infection in cultured trophoblasts was clearly demonstrated by the expression of an early to late expressed tegument protein, pp65, and progeny virus

production. Progression of the infection in trophoblasts as in most other cell types infected *in vitro* (Michelson-Fiske *et al.*, 1975; Knowles, 1976; Tumilowicz *et al.*, 1985; Smith, 1986; Lathey *et al.*, 1990; Poland *et al.*, 1990; Cinatl *et al.*, 1994) lagged behind that of fibroblasts temporally and in terms of infection efficiency and progeny virus levels (compare Figure 6.2 and Table 6.3). The expression of pp65 was obviously delayed in infected trophoblast cultures compared to infected fibroblast cultures and in term +EGF cultures did not appear to reach the same frequency of expression as IE antigen at any measured time point (Figures 4.2 and 4.9). After optimal viral challenge, however, pp65 expression frequency appeared to approach that of IE in infected –EGF cultures but not in infected +EGF cultures at later time points (i.e. Figures 6.2A and 6.3). This suggests that at least some of the IE-positive cells, particularly in +EGF cultures, may be abortively rather than permissively infected (Cinatl *et al.*, 1994). This proposal may also explain the increasing frequency of IE-positive nuclei over time observed in infected –EGF cultures but not in infected +EGF cultures (Figure 6.2A).

Thus comparing total progeny virus produced to total IE-positive cells per well may not be an accurate representation of infection progression, particularly in +EGF cultures (Figure 6.4). There are likely many IE-positive cells that do not progress to the virus production stage and all +EGF data points would shift to the left in Figure 6.4, although the total progeny virus would remain the same. Thus the amount of virus being produced by one permissively infected cell in the +EGF culture would likely be similar to that being produced by one permissively infected cell in the –EGF cultures. This proposal is consistent with the suggestion that the maturation state of the trophoblast and not the culture condition (i.e. the presence or absence of EGF) is important not only for initiation of infection but also for progression to a fully permissive infection.

To confirm that infected cells in either culture are equally productive in terms of progeny virus levels, the same type of analysis could be done using a more specific non-structural late antigen to avoid the complication of detection due to viral entry. Although pp65 entry after initial infection could not be detected, later in infection when cell-to-cell infections occurred with large numbers of progeny virus, both pp65 entry and production could be detected. Thus use of a tegument protein such as pp150 that is undetectable upon viral entry because of its low concentration may be a preferable marker of late

infection (Sinzger *et al.*, 1993). In situ hybridization for viral DNA or viral mRNA could also be used to identify the number of cells progressing to a late stage of viral infection (Schrier *et al.*, 1985; Ozono *et al.*, 1997). Comparing the amount of supernatant virus per viral antigen positive cell has been previously used to compare infection characteristics between HCMV laboratory strains and clinical isolates (Yamane *et al.*, 1983).

Delayed progression of infection and reduced progeny virus release in trophoblast cultures compared to fibroblast cultures were also shown by Halwachs-Baumann et al in a paper published three months after my initial paper (Halwachs-Baumann *et al.*, 1998). As shown by my work, they also found variable susceptibility to HCMV infection with three clinical isolates and two laboratory strains (5 to 50% infection levels). However, they reported infection frequencies up to 100% in their syncytiotrophoblast-like cultures compared to the low levels I observed in +EGF cultures. These cultures progressed to complete lysis after only 14 days, although comparison to uninfected cultures was not mentioned. Possible explanations for the discrepancies are 1) increased contamination with vimentin-positive cells (they report >95% trophoblast purity compared to >99.99% purity in my cultures) may result in high titres of released virus within three days, effectively adding to the initial MOI used and 2) trophoblast isolation procedures are different and the population of cells isolated may differ in many respects including HCMV susceptibility and secretion of inflammatory agents such as TNFα that can mediate apoptotic death of trophoblasts (Yui *et al.*, 1994).

Infection in a rhabdomyosarcoma cell line showed many of the same infection parameters as in trophoblasts: immature rather than differentiated cells could be permissively infected, progression of infection was slow, virus remained predominantly cell-associated and only half as many cells produced late antigen as those producing IE antigen (Cinatl *et al.*, 1994). The spontaneously differentiated multinucleated cells were only able to express IE antigen suggesting, as in trophoblasts, an abortive infection occurred. The authors also showed that small populations of undifferentiated cells found in their more differentiated cultures could be permissively infected even in the presence of more mature cells. This confirmed that the maturation state of a cell was more important to HCMV susceptibility than the particular culture it was growing in and that non-permissively infected cells were likely not secreting antiviral agents.

EGF itself did not appear to negatively affect progression of infection in –EGF cultures (Figure 6.8), confirming that the initial stage of differentiation is important to HCMV susceptibility and not the culture condition in which the cells are found. It remains unknown whether EGF was able to induce differentiation in these cultures regardless of the presence of HCMV, but infection nevertheless progressed in a similar fashion to –EGF cultures. Latently infected $CD34^+$ stem cells were able to differentiate to the more committed granulocyte-macrophage progenitor cells without appreciable viral reactivation suggesting infection may not prevent differentiation (Hahn *et al.*, 1998). Use of another agent such as CSF-1, GM-CSF or phorbol esters to induce differentiation and multinucleation in trophoblast cultures would confirm the importance of the differentiation state and not the agent in susceptibility to HCMV infection (Garcia-Lloret *et al.*, 1994).

Even though trophoblasts in both cultures were permissively infected with HCMV, the majority of cells remained IE-negative or expressed only IE antigen, suggesting either latent or abortive infections. In the hematopoietic system, infection of $CD34^+$ stem cells and the $CD33^+$ granulocyte-macrophage progenitor cells results in latent infections (Kondo *et al.*, 1994; Minton *et al.*, 1994; Hahn *et al.*, 1998). Reactivation by either coculture with HCMV permissive cells (Kondo *et al.*, 1994; Hahn *et al.*, 1998), culture in the presence of inflammatory cytokines (Hahn *et al.*, 1998) or upon stimulation with allogeneic peripheral blood mononuclear cells (Soderberg-Naucler *et al.*, 1997) occurs in the $CD33^+$ granulocyte-macrophage progenitor population. Differentiation in the hematopoietic system appears to be essential to activation of the infection from latency except in the case of the $CD34^+$ stem cells, which retain their latent viral infection even upon differentiation to $CD33^+$ progenitor cells. Perhaps the IE-negative cells in the trophoblast cultures are in fact at a differentiation state similar to the $CD34^+$ stem cells and are thus latently infected These cells may remain undifferentiated and thus latently infected. Detection of latency in these cell populations could be assessed by inducing reactivation of the virus by the methods described above and by using *in situ* PCR to look for viral DNA as well as latency associated mRNA transcripts.

Significantly more cell loss occurred in infected –EGF compared to infected +EGF cultures when compared to their respective uninfected controls after but not before

six days of viral challenge (Figure 6.5). This delay in cell loss corresponds to the difference in the amount of progeny virus produced by –EGF cultures compared to +EGF cultures at 11 days but not 3 days after viral challenge (Figure 6.4B). Progeny virus remained predominantly cell-associated in these cultures and any increase in apical release coincided with increased cell loss (Figures 6.2B and 6.5), suggesting that the released virus may be due in part to cell lysis induced by infection in these cultures. HCMV infections are known to induce bystander effects resulting in loss of uninfected cells by apoptosis (Waldman *et al.*, 1998; Kosugi *et al.*, 1998). To determine if apoptosis contributed to the cell loss observed in –EGF cultures, TUNEL was performed and compared to cell loss. Increasing cell loss as a function of time was associated with increasing numbers of TUNEL-positive cells (Figure 6.6). Cell loss appeared to be greater than the number of TUNEL-positive cells suggesting two possibilities: 1) some infection-induced lysis likely occurs and 2) TUNEL-positive cells in the late stages of apoptosis may be washed away during fixation and washing procedures, leaving only adherent apoptotic cells to be counted.

As HCMV is able to specifically block apoptotic pathways (Zhu *et al.*, 1995; Cinatl *et al.*, 1998; Lukac, Alwine, 1999), it is unlikely that the TUNEL-positive cells observed were also virally infected. It is more probable that HCMV infection was stimulating the infected trophoblast to produce an agent that induced apoptosis in nearby non-infected cells. HCMV infection activates production of a number of cytokines including IL-6 (Iwamoto, Konicek, 1997) and IL-8 (Murayama *et al.*, 1997) as well as various transcription factors such as NF-κB and SP1 (Yurochko *et al.*, 1997). Since the promoter region of TNFα contains sites for binding NF-κB (Shakhov *et al.*, 1990; Drouet *et al.*, 1991; Albrecht *et al.*, 1995) and trophoblasts are known to produce TNFα (Chen *et al.*, 1991; Ghosh *et al.*, 1998), infection by HCMV or in fact just binding of the virus or its glycoproteins alone (Yurochko *et al.*, 1997) may induce production of this cytokine. The apoptotic effects of TNFα on trophoblasts (Yui *et al.*, 1994) through the p55 receptor (Yui *et al.*, 1996), can be prevented by the presence of EGF (Garcia-Lloret *et al.*, 1996). Thus the increasing apoptosis observed in infected –EGF trophoblast cultures could be an

indirect result of virus-induced release of TNFα by infected trophoblasts that induces apoptosis in uninfected bystander cells.

The unusual cell loss observed in Figure 6.7 that was associated with increased numbers of TUNEL-positive cells in infected +EGF cultures but not –EGF cultures is difficult to explain by this same reasoning since EGF protects against TNFα induced apoptosis. Perhaps this particular trophoblast preparation carries specific characteristics that *in vivo* would result in a combination of responses to HCMV that would result in vertical transmission. The disparity of this one preparation suggests that many more trophoblast preparations should be studied with respect to the specific characteristics of susceptibility to HCMV infections. Double immunofluorescence to detect IE and TUNEL-positive nuclei could be used to determine which cell population in a culture is dying by apoptosis, i.e. the infected cells or the uninfected cells. Blocking apoptosis using agents such as anti-TNFα or soluble TNF receptors would begin to delineate the role of TNFα in trophoblast cell loss during infection.

Using the optimal viral challenge protocol I infected trophoblasts with a congenital isolate and found similar results to that found with the laboratory strain AD169 (compare Figure 6.2 to Table 6.2). A slightly higher infection frequency was noted in the +EGF compared to –EGF cultures, a finding opposite that found for AD169, although progeny virus levels remained higher in –EGF cultures. Profound differences in the infection of endothelial cells with fresh clinical isolates compared to laboratory strains with high passage number in fibroblasts have been found (Kahl *et al.*, 2000; Sinzger *et al.*, 2000). Similarly, high levels of infectious virus and viral products were found associated with PMNLs within 60 minutes but only upon coculture with endothelial cells or fibroblasts infected with clinical isolates and not laboratory adapted strains (Gerna *et al.*, 2000). These findings suggest the importance of continued testing of clinical isolates on panels of trophoblast preparations to determine if tropism for trophoblasts exists and if the characteristics of clinical isolate infection differ from my results with a laboratory strain.

9.6 DEVELOPMENT OF A CULTURE MODEL TO TEST BARRIER FUNCTION OF TROPHOBLASTS

Progeny virus remained predominantly cell-associated in infected trophoblast cultures grown on solid substrata and infection in cultures most closely resembling the ST (+EGF cultures) did not appear to result in damage. Thus an investigation into whether these infected epithelial cells could release virus from their basal surfaces in a fetal direction was imperative and constituted the final aim of this thesis.

Development of an effective *in vitro* model of the villous syncytium for studies of placental barrier function has been difficult. Primary CTs are often isolated with high contamination levels of placental fibroblasts and macrophages and cultures are usually not viable for longer than a week. Further, primary villous trophoblasts do not proliferate in culture and tend to form non-adhering aggregates at high seeding densities, making confluent cultures difficult to attain. These problems were overcome by: (a) use of highly purified CT preparations that prevented cellular disruptions by fibroblast overgrowth or macrophage secretions (eg. Vettenranta *et al.*, 1986; Yeger *et al.*, 1989; Yui *et al.*, 1994); (b) multiple cycles of seeding and differentiation which allowed formation of a confluent relatively uniform multilayer of non-proliferating trophoblasts; (c) alternation of seeding and differentiation which minimized loss due to self-aggregation or clump formation; and (d) use of highly viable CT preparations which provided the continuous long-term culture required for generation of confluent layers by alternation of seeding and differentiation. The insert membrane cultures thus prepared were confluent multilayers of syncytial units with tight junctions that formed effective physical barriers limiting transepithelial diffusion of high (^{14}C-dextran) and low (^{3}H-inulin) molecular weight molecules as well as organisms such as HCMV. It is clear that assessment of HCMV diffusion may be complicated by the adherence properties of the virus to +EGF trophoblasts and thus would not be a good measure of culture confluence on its own.

I chose to use a 3.0 μm insert membrane pore size because diffusion of the 0.2 to 0.3 μm virus particles may be restricted through the smaller 0.45 μm pores. Five times more HCMV was required to infect non-polarized fibroblasts grown on inserts with 0.45 μm pores from the lower chamber than from the upper chamber (Tugizov *et al.*, 1996),

demonstrating that some restriction through smaller pores occurs. This restriction through 0.45 μm pores could be overcome by using centrifugation of the virus onto inserts. Preferential infection in tight-junctioned Caco-2 cells through the basolateral membranes was shown by culturing the cells on the bottom of the filter membrane so that the basolateral membrane was exposed to the upper reservoir of the filter. Addition of HCMV to the upper reservoir followed by centrifugation resulted in 20% IE-positive cells compared to 2% if cells were infected in a similar manner when cultured in the upper reservoir (apical surface; Esclatine *et al.*, 2000). Another study demonstrated preferential basolateral HCMV infection of Caco-2 cells through inserts with 3.0 μm pores and progeny virus could be detected in the basal compartment late in infection (Jarvis *et al.*, 1999). I confirmed that HCMV could move through the 3.0 μm pores of the insert filter membrane by applying the virus to the apical chamber of a cell-free insert and monitoring diffusion to the basal chamber (Figure 7.1 and Table 7.1).

Epithelial cells such as MDCK II replicate in culture and form well-defined tight-junctioned monolayers (reviewed in Simons, Fuller, 1985) to which I compared the triple-seeded trophoblast cultures. The TER of 12-day triple-seeded trophoblasts was higher than that of confluent monolayered MDCK II cells, reflecting not only the presence of tight junctions but also the multiple layers of large syncytialized cells (Table 7.1). Even so, the confluent MDCK II cells created a more effective barrier to passive transepithelial diffusion of either ^{14}C-dextran or HCMV than triple-seeded trophoblasts after 12 days of culture. A barrier as effective as that produced by MDCK II cells (0.286 ± 0.170 pmoles/hr/cm^2) was only detected in 32-day trophoblast cultures with a TER > 75 ohms x cm^2 (Figure 7.5). Small holes undetectable by light microscopy but present in 12-day cultures may close during extended culture resulting in improved barrier function. Alternatively, no holes may exist and the tight junctions present between syncytia may simply mat[illegible] increase in frequency and begin to approach the efficiency of those in MDCK II cultures. Increases in TER have been observed upon treatment of LLC-PK$_1$ epithelial cells with EGF, which the authors suggest may be a result of a change in the charge selectivity of the tight junctions (Soler *et al.*, 1993). Since trophoblast cultures are continuously treated with EGF, a combination of charge selectivity and maturation may

result in the observed increase in TER and decrease in passive diffusion. The predictability of an inverse relationship between transepithelial diffusion and TER in triple-seeded trophoblast cultures allows either measurement to be used to monitor barrier function.

Although I have solved some of the difficulties observed with discontinuous membrane cultures such as the large leak factor (Bullen *et al.*, 1990; Sooranna, Contractor, 1991), the multiple overlapping layers of syncytial trophoblast units over the majority of the insert membrane surface may limit the use of these cultures in their present form for studies of facilitated transport. The multiple layer model, however, complete with a basement membrane, may be more physiological in other aspects than a single layer of trophoblasts since *in vivo*, particularly in first trimester, there are two complete layers of trophoblasts. Further, my finding that trophoblast membrane and cytoplasm can extend through 3 μm pores approximates the situation *in vivo* where pseudopods of ST are often seen to move into the TBM (Ashley, 1965). Finally, preferential basal release of MMP-9 demonstrates functional apical/basal differentiation.

9.7 PROGENY VIRUS REMAINS PREDOMINANTLY CELL-ASSOCIATED IN INFECTED TROPHOBLAST BARRIER CULTURES

The final part of my hypothesis states that once infected, trophoblasts release progeny virus in a basal direction. Using the barrier culture model defined in the previous section, I was now able to test this hypothesis. I was unable to detect progeny virus in the basal compartment from these cultures despite the presence of cell-associated virus (Figure 8.1). A number of problems make interpretation of this result difficult. The infection efficiency of triple-seeded cultures was low (e.g. Table 8.1) as might be expected from the results shown in Figure 5.5A where length of time in culture reduced susceptibility of trophoblasts to infection. The multilayered nature of these cultures (average of 2.7 cells; Figure 7.8) is such that an infection of the uppermost layer may not progress through to the layer directly in contact with the insert surface, although infection clearly progresses laterally in trophoblast cultures (Figures 4.2B and 4.5). To address these questions, I first showed that although infection frequency was low, pp65-positive nuclei could be detected in direct contact with the insert membrane by confocal

microscopy (Figure 8.3). This suggested that basally released virus from these infected cells could potentially reach the bottom chamber (however, see later discussion on the role of TBM). This also demonstrated that the trophoblasts in these cultures were layered in such a way that inoculum virus was able to reach the cells in direct contact with the filter or that cell to cell lateral progression from the uppermost infected cells could occur.

To prove unequivocally that HCMV infection existed in the layer of cells directly attached to the filter, I modified the protocol developed in Chapter 7.0. Instead of challenging the cultures with virus after the third and final seeding of trophoblasts, I challenged the first seeding after three days in culture with EGF and overlaid the infected cells with two more seedings of trophoblasts as described in the original method. This altered protocol not only ensured the presence of infected cells in the bottommost layer, it also increased the infection efficiency dramatically (compare Figure 8.3 to Table 8.1). Even so, the results obtained confirmed the original finding that little progeny virus could be found in the basal compartment (Figure 8.3C).

A summary of four experiments performed with the modified culture technique (Figure 8.4) demonstrated clearly that the majority of progeny virus produced in these cultures either remained cell-associated or were trapped between the trophoblasts and the TBM. To allow the infection to progress as long as possible, analysis for basal release was done just prior to the point of culture breakdown as detected by a decrease in TER and an increase in ^{14}C-dextran diffusion. The number of days after viral challenge on which analysis was done varied for each experiment and trophoblast preparation. The median number of infectious virions detected in the bottom chamber was approximately 1100 times less than that found cell-associated and 170 times less than that found in the apical supernatant. These values are likely underestimated because although virus is directly measured in the bottom chamber, apical and cell-associated aliquots are frozen and thawed once and thrice respectively before assay on fibroblasts. Approximately 10% of viral activity is lost during each freeze thaw cycle.

The rate of virus diffusion through a cell-free insert is likely higher than the reported 10.9%/hr (Figure 7.1) due to loss from heat inactivation of more than 70% within two hours at 37°C (Vonka, Benyeshmelnick, 1966). Thus this virus is able to diffuse through the insert pores in a somewhat restricted manner likely due to heat

inactivation, adherence to the insert surface and the time required for movement from the upper medium to and through the insert pores. Two of these restrictions are reduced in the situation where infected trophoblasts are in direct contact with the insert membrane: the virus would be protected from heat inactivation until released and upon release may move directly through the adjacent pores. Virions released in-between pores may be caught between the cell surface and the insert surface and would not be detected in the basal chamber (see the following discussion for implications involving the TBM). Even if half of the virus basally released is retained in this manner, only an average of 0.14% of total progeny virus would be detected in the basal compartment.

It is possible that even the small amount of virus detected in the basal compartment could be due to leakage of apically released progeny virus through holes or breaks in the cell layers. Almost all infected fibroblasts found in the lower chamber were detected in an outer ring that mirrored the edge of the culture insert. Breakdown of the insert cultures almost invariably begins at the outer edge leaving the center of the culture intact. HCMV-infected Caco-2 epithelial cells grown on 3.0 μm insert membranes showed similar results with most virus remaining cell-associated, some apical release and no basal release observed until after the cellular monolayer began to deteriorate (20 days after viral challenge; Jarvis *et al.*, 1999).

These studies suggest that transmission of the virus to the fetus as a result of infection alone is unlikely. However, I am unable at this point to conclusively state whether the lack of detected virus in the bottom chamber is due to the lack of basal release from the infected trophoblasts or due to the presence of an intact TBM functioning as a barrier. As previously discussed an intact electron dense layer, likely the TBM, between the trophoblast and the insert membrane can be seen in some cases covering but not entering the pores. It has recently been shown that one component of the TBM is perlecan, a heparan sulphate proteoglycan (Rohde *et al.*, 1998), to which HCMV can bind. The TBM could be envisioned to act as a sponge, mopping up free virus until removed by fetal macrophages or Hoffbauer cells. Electron microscopy could be used to determine if progeny virus is sequestered in vacuoles in the trophoblast as was found in macrophages (Fish *et al.*, 1995) or released and trapped in the trophoblast basement membrane. Burton (Burton, Watson, 1997) suggests that the basement membrane may

play an important barrier role in the placenta since transient damage down to the TBM followed by repair is often observed throughout gestation without concomitant fetal damage (Burton *et al.*, 1996; Watson, Burton, 1998). Lack of basal release from infected trophoblasts and/or presence of the TBM acting as an effective barrier may explain why vertical transmission does not appear to occur more frequently in the first than third trimester (Kumar, Prokay, 1983; Cook *et al.*, 1993) even though first trimester trophoblasts are more readily infected. These observations suggest insert membrane cultures as developed in this study are an important first step in the development of more physiological *in vitro* models of the villous placenta. Such models are essential for studies of barrier function, transport and metabolism of drugs and nutrients and placental cell-to-cell interactions.

9.8 RELEVANCE TO *IN VIVO* SITUATION

Villous trophoblast infection if it occurs *in vivo* obviously does so at a level difficult to detect using conventional sampling techniques of placental tissue (Benirschke *et al.*, 1974), e.g. it is probably an infrequent event at best. However, a small number of focal events may be enough to lead to further placental and fetal infection. To begin to understand a limited number of events, demonstration and follow-up of infection in cultured trophoblasts required an unnatural enhancement of infection. Thus most of the experiments described in this thesis use a high ratio of virus to cells. This ratio is similar to that used by other investigators (Amirhessami-Aghili *et al.*, 1989; Fisher *et al.*, 2000). In some cases, low MOIs reportedly used were based on total number of cells seeded in culture, not the number of adherent cells at the time of viral challenge, which would underestimate the actual MOI used (Toth *et al.*, 1995a; Toth *et al.*, 1995b). All studies in this thesis used MOIs determined by using careful estimates of the number of adherent cells. Adherence of trophoblast preparations can vary widely from 10% to 50% and it is therefore likely that the MOI of 10 used in these studies is similar to the actual MOIs used in these other studies.

Additionally, the sub-optimal use of cell-free virus rather than a more physiological cell-to-cell mechanism of viral challenge is acknowledged. The results in this thesis should be confirmed by using infected monocytes (Taylor-Wiedeman *et al.*,

1991; Guetta *et al.*, 1997), granulocyte progenitors (Minton *et al.*, 1994; Hahn *et al.*, 1998), PMNLs (Revello *et al.*, 1998; Gerna *et al.*, 2000) or endothelial cells (Waldman *et al.*, 1995; Fish *et al.*, 1998) in coculture with trophoblasts or alternatively by using infected cells from viremic patients.

In vivo even viremic patients have levels of HCMV positive cells less than 1:500 and detection of infected cells in latency or with low-grade persistence is difficult. Viremia, pp65 antigenemia and leukoDNAemia in maternal blood do not seem to predict or correlate to intrauterine transmission of HCMV (Revello *et al.*, 1998). However, even in cases of transmission involving transfusion or transplantation, only low to almost undetectable levels of actively infected cells can be found. Thus, it is still relevant to assume that hematogenous transmission via the placenta could also occur even with demonstration of low levels of infected cells in maternal blood.

9.9 A MODEL OF HCMV PLACENTAL INFECTION LEADING TO FETAL INFECTION

Primary villous trophoblasts isolated from first trimester or term placentas can be permissively infected with HCMV. My *in vitro* results are consistent with the more frequent and later HCMV infection stages found in first trimester (Garcia *et al.*, 1989; van Lijnschoten *et al.*, 1994) than term (Quan, Strauss, 1962; Monif, Dische, 1972; Otto *et al.*, 1988; Garcia *et al.*, 1989; Muhlemann *et al.*, 1992; Sinzger *et al.*, 1993; Nakamura *et al.*, 1994) ST *in situ*. However, *in vivo* detection of HCMV-infected ST is less frequent than would be anticipated given the more frequent *in vivo* observations of infected fetal stromal cells and my *in vitro* observations of higher susceptibility and infection progression in CT-like rather than ST-like trophoblasts. The *in vivo* and *in vitro* observations can be reconciled by three possible explanations: (a) Virus titers in the maternal circulation are not high enough to infect the ST or the ST is relatively resistant to infection and the virus enters (perhaps via HCMV-infected maternal leukocytes) the stroma through breaches in the ST caused by physical trauma or at sites of trophoblast damage caused by intervillous accumulations of activated monocytes (intervillositis; Jacques, Qureshi, 1993), (b) The ST is infected at sites of recently fused CT that occurs as often as infection in the stroma but progeny virus is retained and manifestations of ST

infection are rapidly lost possibly because the infected ST is sloughed off into maternal circulation through normal trophoblast turnover (Huppertz *et al.*, 1999) or (c) Stromal infection does not originate through infection or damage of the villous ST from maternal blood. Rather, CTs that invade the uterine wall are infected by the resident maternal cells and this infection then progresses in a retrograde manner via cell column CTs to the villous stroma as recently proposed (Fisher *et al.*, 2000).

The trophoblast, like other epithelia, would be expected to periodically renew its outer surface. Huppertz et al in recent studies have suggested that the fusion of CTs to existing ST begins a pathway of trophoblast turnover that takes about 26 days to complete and ends with the shedding of apoptotic knots into the maternal circulation from the syncytium (Huppertz *et al.*, 1998; Huppertz *et al.*, 1999). My *in vitro* results show that infection of +EGF cultures (ST-like) retain progeny virus without concomitant cell damage for at least 20 days. As previously seen in guinea pigs (Griffith *et al.*, 1985), the trophoblast may be acting as a sink for the virus, retaining it until the trophoblast is sloughed off into maternal circulation through normal trophoblast turnover (Huppertz *et al.*, 1999). An ST infection in the absence of any other confounding variable would then be of no consequence, the layer being removed and renewed continually. However, because of shedding, the steady state level of obviously infected ST may be low even though infection occurs frequently. Thus, my data, combined with published data, tentatively describe the ST as an infectable barrier that may maintain its integrity by retaining infectious virus until shed.

Based on the results presented in this thesis, i.e. low susceptibility to infection in +EGF cultures and the cell-associated nature of progeny virus, an infection mechanism alone is unlikely to explain how this virus reaches the fetus. Two possible consequences of an infected ST however, suggest that transmission through the villous ST may still occur. If HCMV infection upregulates ICAM-1 (which is inducible in ST; Xiao *et al.*, 1997) as it does in fibroblasts (Grundy, Downes, 1993; Ito *et al.*, 1995b), T lymphocytes (Ito *et al.*, 1995a) and endothelial cells (Sedmak *et al.*, 1994; Waldman, Knight, 1996), infected ST may be preferentially cleared through phagocytosis or TNF-α mediated damage by LFA-1-activated monocytes that adhere to sites of infection. My laboratory

has evidence for damage induced through secretion of TNFα by activated monocytes adhering to ST expressing high levels of ICAM-1 (Garcia-Lloret *et al.*, 2000).

An alternative consequence of an infected ST is the subsequent lateral infection of tight-junctioned underlying CTs. My data suggests that relatively immature trophoblasts are more susceptible to HCMV infection than the ST and that infection is often detrimental in the latter cultures. I have also observed lateral or cell-to-cell infection both in solid phase cultures and through the layers in triple-seeded insert cultures. Destructive infection of underlying CT cells resulting in depletion of the stem cells renewing the ST could lead to focal breaks that allow either infected maternal cells or free virus to enter fetal tissues.

The majority of studies looking for evidence of HCMV infection in placental sections were done by histologically searching for inclusion bodies (Table 1.1; Blanc, 1961; Quan, Strauss, 1962; Hayes, Gibas, 1971; Altshuler, McAdams, 1971; Monif, Dische, 1972; Benirschke *et al.*, 1974; Mostoufi-Zadeh *et al.*, 1984; Garcia *et al.*, 1989). Recent techniques including immunofluorescence or immunohistochemistry along with *in situ* hybridization or PCR have been used and often find evidence of infection in the absence of histological evidence (Garcia *et al.*, 1989; Schwartz *et al.*, 1992; Muhlemann *et al.*, 1992; Sinzger *et al.*, 1993; Nakamura *et al.*, 1994; van Lijnschoten *et al.*, 1994; Schneeberger *et al.*, 1994; Saetta *et al.*, 1998). I found that infection in +EGF (ST-like) cultures did not appear to be lytic and as such infection of the ST *in vivo* may not progress to the point of seeing characteristic inclusion bodies even though it is clearly permissively infected. A similar observation has been made in HCMV infection of cultured human brain cells where typical inclusions and viral antigens were not found even in the face of extensive cytopathology (Pulliam, 1991). This again argues for an indirect effect of infection that may not be immediately recognizable.

One model of vertical transmission that has been proposed (Fisher *et al.*, 2000), suggests that infection originates in the maternal uterine cells and progresses in a retrograde manner through the CTs of the cell column up into the stroma. The authors provided some *in vivo* and *in vitro* evidence for preferential infection of first trimester CT, although infection of the ST was also occasionally detected. In first trimester organ culture, Palmer et al demonstrated syncytial degeneration followed by regeneration from

fusion of underlying CTs by 48 hours (Palmer *et al.*, 1997). Thus it is possible that prior to infection of the explants in Fisher's studies at 12 hours of culture that some of the syncytial layer has degenerated allowing the virus access to CTs without evidence of ST infection. As well, I note that in 5 out of 12 placentas examined, neither CT nor ST infection could be found, suggesting that perhaps in these cases the ST remained intact and acted as a barrier. However, the evidence of CT infection in the absence of ST infection found in placental sections by Fisher et al strongly suggests that this route of transmission does occur. Evidence for deciduitis has been clearly shown but often without accompanying cytomegalic changes (Garcia *et al.*, 1989) and 15% of women with primary HCMV infections have been reported to abort spontaneously (Griffiths, Baboonian, 1984). In one study looking at karyotyped abortions positive pp65 staining in the decidua was strongly correlated with placental and fetal infection and 35% of endometrial biopsies were HCMV positive (van Lijnschoten *et al.*, 1994). Although another large study was unable to identify HCMV DNA in chorionic villi and membranes of 350 spontaneous abortions, no decidual tissue was tested (Putland *et al.*, 1990). HCMV DNA has also been isolated from uterine glandular epithelial cells (Furukawa *et al.*, 1994) and endometrial glands (Dehner, Askin, 1975). This route of transmission is consistent with my data showing increased susceptibility of –EGF (CT-like) compared to +EGF trophoblasts (ST-like). However, it is unlikely to be the only route since decidual infection has not been identified in many cases where chorionic villi are clearly infected (Monif, Dische, 1972). A number of other studies were unable to detect HCMV in the spontaneous abortions studied (Boue *et al.*, 1966; Cook *et al.*, 1993; Sifakis *et al.*, 1998).

My studies suggest that susceptibility and progression of the HCMV infection is dependent on the state of differentiation at the time of viral challenge rather than the presence or absence of EGF. This particular state of differentiation may be more prevalent in –EGF and young +EGF cultures. Morrish et al have demonstrated that –EGF trophoblasts isolated using similar protocols to those used in this thesis, have already begun to differentiate as early as 24 hours in culture (Morrish *et al.*, 1997). Thus *in vivo* these susceptible cells may correspond to mononucleated CT that have reached a maturation state just prior to fusion or have recently fused corresponding to HCMV susceptible focal areas of the ST. Therefore, my current hypothetical model (Figure 9.1)

of infection events occurring at the ST is the following: **(A)** An infected leukocyte or cell-free virion enters the intervillous space in maternal blood and comes into contact with a susceptible focal area of the villous ST. **(B)** A localized permissive infection ensues but **(1)** is retained by the ST which eventually progresses towards apoptosis and is sloughed off or **(2)** is transmitted laterally to a tight-junctioned differentiating CT. The possible fates of the infected CTs are shown in Figure 9.1C. Infected CTs could fuse back into the ST early in infection, in which case the resultant progeny virus may be retained by the ST as in (1) until it is eventually sloughed off into maternal circulation (not shown on diagram). **(3)** The infected CT may undergo infection-induced lysis prior to fusion, releasing progeny virus, which may be retained by an intact TBM until the ST is sloughed off. **(4)** Even if progeny virus is prevented from entering the stroma by the TBM, the focal loss of CT stem cells as a result of infection-induced cell lysis or apoptotic bystander effects of inflammatory cytokines such as TNFα may result in a thinning of the ST in the infected area. This could induce focal damage leading to breaks in the ST, increasing the possibility of stromal entry by infected maternal leukocytes or cell-free virus. Even so, a combination of natural trophoblast repair mechanisms and the presence of an intact TBM may preclude transmission by this route (Burton, Watson, 1997).

Although it is possible that HCMV may cross the villous placenta as described in Figure 9.1, it is equally possible that the route proposed by Fisher et al (Fisher *et al.*, 2000) could be operating. Since the route across the ST of the floating villi necessarily involves direct contact with maternal blood, it is interesting to postulate that this route may be important during primary infections in which viremia occurs (Revello *et al.*, 1998). During a reactivated infection in which the incidence of infected maternal blood cells is either low or unlikely, a reactivated infection originating in the uterus may be the most important possible route of vertical transmission (Fisher *et al.*, 2000).

9.10 FUTURE DIRECTIONS

My results argue that permissive infection of villous ST or CTs in late gestation by cell-free HCMV can occur but only if the viral titer in the maternal circulation is very high. Such levels could occur during primary infections because of the transient absence

of neutralizing antibody and may partially explain why vertical transmission is much more frequent in primary than recurring infections (Yow *et al.*, 1988; Boppana, Britt, 1995). It is more likely, however, that the infection is present in maternal leukocytes, which makes it imperative to study the role of cell-to-cell transmission of HCMV to trophoblasts. Recent development of techniques to isolate viable infected leukocytes from patients with viremia and coculture techniques to activate a latent infection in monocytes (Waldman *et al.*, 1995; Guetta *et al.*, 1997) will provide a source of infected cells to test on insert cultures with confluent trophoblasts. A number of criteria could be examined: trophoblast infection characteristics and progression, induction and mechanism of trophoblast damage by the infected monocyte and movement of infected monocytes through the trophoblast culture to the bottom chamber.

Although trophoblasts cultured with or without EGF can be permissively infected it appears that the majority of cells even at late stages of infection remain resistant as determined by lack of IE-antigen expression. Additionally, in +EGF cultures far more cells express IE antigen than pp65 antigen suggesting that many of these infected cells are abortively infected, never progressing beyond the IE-antigen stage. At what stage are trophoblasts able to resist infection: entry, nuclear translocation, IE-antigen expression? Are some of these cells latently infected? What characteristic(s) of non-infected cells induce(s) resistance? Although my results point to a particular differentiation state that may be important to HCMV susceptibility, defining what it is about a particular state that confers resistance would be an important question to address. Each of the above infection stages can be monitored using various techniques: entry as previously described using a recombinant HCMV expressing green fluorescent protein (GFP; Vieira *et al.*, 1998), nuclear translocation by competitive HCMV-DNA-PCR of nuclear fractions (Sinzger *et al.*, 2000) and specifically blocking or activating the IE promoter region with an attached reporter gene that has been transfected into the cells (Liu *et al.*, 1994). Whether some of the non-infected trophoblasts have actually entered latency could be monitored by using in situ hybridization or PCR to detect copies of viral DNA (Kondo *et al.*, 1994; Kondo, Mocarski, 1995; Ozono *et al.*, 1997; Saetta *et al.*, 1998). Alternatively, various studies have shown that coculture with a permissive cell may induce abortively or latently

infected cells to enter the lytic phase (Waldman *et al.*, 1995; Guetta *et al.*, 1997; Bacsi *et al.*, 1999).

I have optimized trophoblast viral challenge protocols to achieve appreciable infection levels using the laboratory strain of HCMV, AD169. Only one clinical isolate (Kp7) was tested using the improved challenge protocols. Studies looking at HCMV infection in endothelial cells (Kahl *et al.*, 2000) and monocyte-derived dendritic cells (Riegler *et al.*, 2000) suggest that clinical isolates and laboratory strains of virus behave very differently with respect to infection, progression and cellular outcome. Importantly, continued passage through fibroblasts seems to alter the virus's original tropism (Brown *et al.*, 1995; Cha *et al.*, 1996). Sinzger et al recently found that differences in endothelial cell tropism for various HCMV isolates was due to differences in the efficiency of transport of capsids to the nucleus (Sinzger *et al.*, 2000). Monitoring susceptibility and infection progression of a large number of clinical isolates from infants with congenital infections on a large panel of first trimester and term trophoblast preparations will begin to determine the importance of tropism to vertical transmission of HCMV. Determining the step at which some but not all isolates are blocked from trophoblast infection may suggest treatment regimens.

I was unable to demonstrate the accumulation of appreciable progeny virus in the basal compartment from infected trophoblasts grown on culture inserts. Although the majority of progeny virus appear to be cell-associated the possibility remains that virus could be released basally from the trophoblasts but accumulate between the cell and the TBM. As previously mentioned, using the electron microscope to look for the location of virus particles would delineate between the two possibilities. As well, these studies do not address the possibility of cell-to-cell transmission to underlying fetal stromal cells, whether by release and uptake through the respective basement membranes or through direct cell-to-cell contact. *In vivo*, particularly at term, the TBM of vasculosyncytial areas of the syncytium is often directly apposed to the basement membrane of fetal endothelial cells (Fox, Agrafojo-Blanco, 1974). Trophoblast pseudopods protruding into the basement membranes may facilitate cell-to-cell transmission (Chapter 7.2.5; Ashley, 1965). Further development of the insert culture model to allow trophoblasts cultured on the inner surface of the insert to directly contact fetal endothelial cells or fibroblasts

cultured on the bottom insert surface will be crucial to further understanding not only of viral transmission but of cross-talk occurring between integral placental cells.

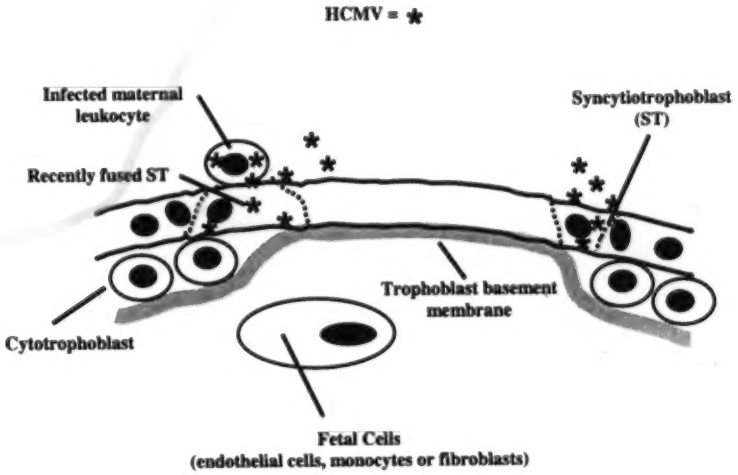
The ST is active in producing many hormones, enzymes and proteins important to the survival of the fetus (reviewed in Castellucci, Kaufmann, 2000). Although there appears to be an excess of CTs, any interaction that negatively affects the function and vitality of these cells may negatively impact the fetus as well. HCMV infected extravillous trophoblasts clearly show reduced invasion capability suggesting a possible role in shallow implantation as observed in diseases such as preeclampsia (Fisher *et al.*, 2000). Using the culture and viral challenge systems developed in this thesis, further studies into the effects of HCMV infection on normal trophoblast function including hormone and enzyme production, fusion characteristics, apoptotic susceptibility and transport systems will further our understanding of related problems during congenital HCMV infections such as intrauterine growth retardation (Stagno, 1995).

Trophoblasts generate IFNs in response to infection (Aboagye-Mathiesen *et al.*, 1995) and it is entirely possible that many of the non-infected cells within a trophoblast culture are simply protected by their presence. Interestingly, cultured multinucleated cells appear to produce more IFNα than mononucleated cells (Aboagye-Mathiesen *et al.*, 1995) which could explain why my +EGF cultures are permissively infected at lower levels than my –EGF cultures. This innate protection mechanism should be evaluated in response to HCMV infection. The binding of the virus envelope proteins induces the expression of IFN responsive genes in that cell. If infection occurs, this response is eventually downregulated (Miller *et al.*, 1999; Miller *et al.*, 2000). However, if no infection occurs, i.e. through binding of a non-infectious particle or a dense body, these upregulated genes may confer protection of the cell against true infection. Investigations using glycoprotein B or purified dense body preparations followed by infection with purified virus (by banding on density gradients), and determination of a time course of upregulated genes may yield some interesting results.

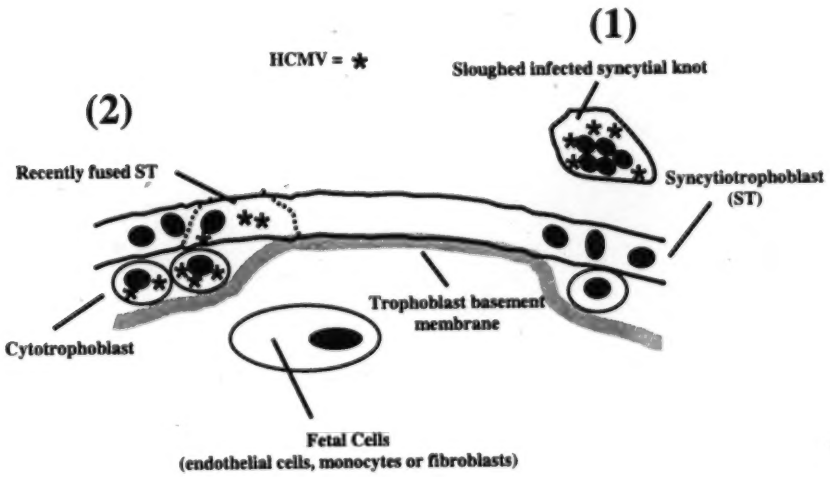
One of the advantages of using these primary trophoblast cultures is the heterogeneity of differentiation states observed within each culture (Figure 5.7, Table 5.1 and Kilani *et al.*, 1997). The availability of trophoblasts in a continuum of maturation from immature to terminally differentiated provides a unique system to investigate

susceptibility to HCMV. A further advantage may be the multilayered nature of both solid substratum and insert membrane cultures. If these cultures are composed of an upper multinucleated layer lying over primarily mononucleated cells then this clearly represents the *in vivo* situation. The disadvantage of heterogeneity in primary trophoblast cultures is that markers to define specific differentiation states in relation to the *in vivo* situation are in their infancy (Huppertz *et al.*, 1998; Benirschke, Kaufmann, 2000a). Along with PLAP expression and syncytialization, markers of apoptosis may provide some information as to the maturation state of trophoblasts, including monitoring transcriptional activity by measuring the incorporation of ^{3}H-uridine into RNA. Continued investigations into the specific state of differentiation that is most susceptible to HCMV infection should yield valuable information with respect to mechanism of vertical transmission and possible methods of intervention.

A


HCMV = *
Infected maternal leukocyte
Syncytiotrophoblast (ST)
Recently fused ST
Trophoblast basement membrane
Cytotrophoblast
Fetal Cells
(endothelial cells, monocytes or fibroblasts)

B


(1)
HCMV = *
Sloughed infected syncytial knot
(2)
Recently fused ST
Syncytiotrophoblast (ST)
Trophoblast basement membrane
Cytotrophoblast
Fetal Cells
(endothelial cells, monocytes or fibroblasts)

C

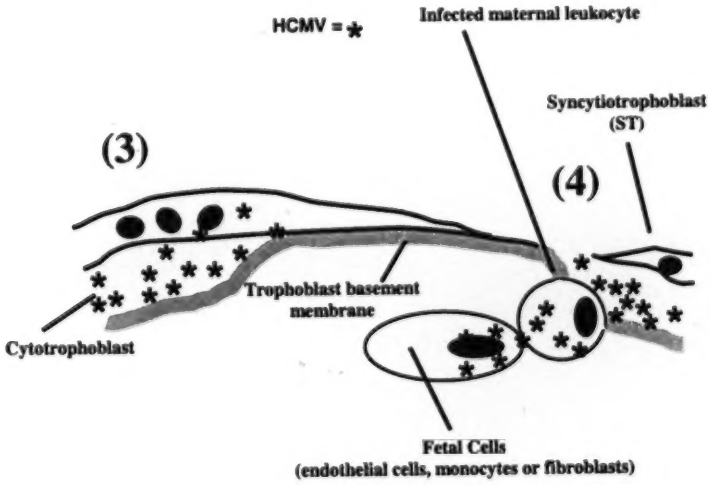

HCMV = *
Infected maternal leukocyte
Syncytiotrophoblast (ST)
(3)
(4)
Trophoblast basement membrane
Cytotrophoblast
Fetal Cells
(endothelial cells, monocytes or fibroblasts)

Figure 9.1 Hypothetical model of vertical transmission through the villous syncytiotrophoblast.
(A) Focal areas of the ST corresponding to areas of recent fusion are infected by cell-free or cell-associated HCMV. **(B)** (1) The infection is retained by the ST and is eventually sloughed off into maternal circulation or (2) the infection is transmitted to the underlying susceptible CTs. **(C)** The possible fates of CTs are: (3) CTs lyse as a result of infection-induced cell lysis but progeny virus is contained by the TBM or (4) Loss of CT stem cells through infection-induced cell lysis or indirectly by induction of apoptosis results in a thinning of the ST in that area. This leads to focal damage and breaks through which infected maternal leukocytes or cell-free virus may enter the villous stroma.

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